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13. ABSTRACT (Maximum 200 Words) The major objective of this project was to establish a new modality for the treatment of breast cancer that employs the combination of chemokines with breast tumor-pulsed dendritic cells to both recruit and/or concentrate from the periphery low frequency immune reactive T cells as well as to potently stimulate these effector cells once localized at the vaccination site. This final report documents our successes in a number of areas, both experimentally and clinically. We have completed the overall project, which has resulted in twenty-nine publications, the awarding of a new spin-off NCI/NIH R01 clinical research grant and a T32 training grant in translational tumor immunology and immunotherapy, as well as the initiation of a phase II clinical trial in advanced breast cancer patients. During the course of our studies related to the Technical Objectives, we made the important discovery that SLC can significantly inhibit the growth of breast tumor in mice. We successfully constructed a recombinant adenovirus vector containing the SLC gene, which can transduce both animal and human dendritic cells at high efficiency for use in our cancer vaccine strategy. A second vaccine clinical trial in breast cancer patients to incorporate SLC-producing dendritic cells is planned.

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FOREWORD

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INTRODUCTION:

The clinical relevance of immunotherapy for the treatment of cancer is premised on the ability to generate a specific cellular immune response against tumor antigens (1). Unlike conventional treatments for tumors (chemotherapy, radiation or surgery) immunotherapy offers the ability of the patient's own immune system to retard tumor growth. However, the weak immunogenicity of some tumors, induction of tolerance by the tumor (2), and the risk of autoimmunity hamper the clinical efficacy of immunotherapy. Some of the treatment regimens designed to elicit anti-tumor immunity include the administration of immunostimulatory cytokines (3, 4), adoptive transfer of in vitro stimulated tumor-reactive T cells (5-8), and administration of antibodies against tumor-associated antigens (9, 10).

Therapeutic cancer vaccines targeted against tumor-associated antigens represent another approach to immunotherapy. Two necessary properties involved in generating tumor vaccines are the presence of immunodominant epitopes expressed by tumors and the presentation of tumor-associated antigens by professional APC (11, 12). Presentation of these antigens on MHC class I or class II molecules by APC expressing co-stimulatory molecules such as B7 and ICAM-1, can initiate an antigen-specific T cell response which, potentially, leads to tumor rejection. Vaccines against defined tumor antigens have been formulated using purified peptides in adjuvant, recombinant viral and bacteria preparations and naked DNA vectors (13). While each of these vaccine approaches have unique properties that make them attractive candidates as therapeutics, they all have the potential problem of limiting the primary immune response against a single tumor antigen. If tumor cells down-regulate expression of the protein used as the immunogen, activated T-cells may be unable to recognize and remove those tumor cells. Another potential problem of these vaccine strategies is the efficacy of antigen uptake and presentation by APC.

Dendritic Cells (DC) are potent APC capable of eliciting a primary immune response to foreign antigens (14, 15). DC strongly express both MHC class I and class II molecules as well as the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2). DC are capable of efficient antigen uptake and priming of naïve CD4 and CD8 T-cells. Large numbers of DC can be readily cultured in vitro from precursors present in bone marrow, blood, and peripheral lymphoid organs (16-18). Because of these properties, DC are attractive candidates for use as APC in therapeutic tumor vaccines.

Preclinical research in our laboratory (19, 20) and in others (21-23) has shown that mice immunized with tumor lysate-pulsed DC (TP-DC) are protected from a subsequent challenge with a lethal dose of viable tumor cells. DC pulsed with lysates from MCA-207 fibrosarcomas are able to generate a tumor-specific cytotoxic T-lymphocyte (CTL) response in vivo (19). Immunization of either BALB/c or C57BL/6 mice with syngeneic DC pulsed with lysates from either MT-901 mammary carcinoma or MCA-207 fibrosarcomas, respectively, resulted in protective immunity from tumor challenge (19). In a therapeutic setting, TP-DC could retard the growth of established subcutaneous breast tumors and fibrosarcomas, although complete remission did not occur (19). Systemic administration of IL-2 along with the TP-DC, however, resulted in dramatic anti-tumor responses in mice harboring either subcutaneous or metastatic tumors (20). These studies suggest that the presentation of tumor antigens by DC, in the presence of immunostimulatory cytokines, results in clinically successful treatment of established tumors. ·

Initial clinical trials involving DC-based immunization of patients with tumors of hematologic and solid origin are promising: subjects show increased antitumor T cell reactivity and experience partial or complete clinical responses (24-29). In one study, treatment of melanoma patients with autologous DC loaded with either tumor lysates or peptide fragments of tumor antigens, resulted tumor specific immunity in 11 of 16 patients and a positive clinical response in 6 of 16 patients (2 complete remissions) (24). In a pilot study of B cell lymphoma, 3 of 4 patients receiving infusions of DC pulsed with antigen (idiotype of tumor Ig) showed an anti-tumor response (2 complete remissions) (25). In a larger lymphoma study by the same group

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(26), 20 of 41 patients treated with the DC vaccine showed an antitumor response with 2 complete remissions. There was no evidence of autoimmunity in patients involved in either the melanoma or lymphoma trials. The results from both of these clinical trials suggest that the response to a DC vaccine, while promising, does not result in successful outcomes in the majority of patients. Diminished clinical efficacy may be due in part to incomplete trafficking of DC to peripheral lymphoid organs. Following s.c. immunization, the majority of DC remain in the injection site and do not traffic to draining lymph nodes or spleen, suggesting that a large number of DC may not be involved in priming a T cell antitumor response (30). It is likely that modification of DC-based vaccines resulting in increased T cell/DC interaction will result in improved clinical outcomes.

Chemokines are a growing family of small cytokines with potent chemotactic activity towards effector cells of the immune system, which are essential for leukocyte trafficking and inflammatory processes (31). These molecules are divided into 4 classes - C, CC, CXC and CX_3C - based on the position of conserved cysteine residues in their sequences and bind to 7 transmembrane receptors on the surface of target cells. Chemokines have been shown to elicit or improve an antitumor response. In the 3LL lung carcinoma model in C57BL/6 mice, DC pulsed with a peptide fragment of Mut1 provided protective immunity to a lethal tumor challenge only when the DC were previously transduced with an adenoviral vector coding for the C chemokine Lymphotactin (Lptn) (21). In a therapeutic setting, Mut1-pulsed Lptn-DC were able to markedly reduce pulmonary metastases, as measured by total lung weight; however, treatment did not lead to complete remission (21). In BALB/c mice, growth of A20 leukemia was severely inhibited when these tumor cells were admixed with Lptn transfected fibroblasts but not vector transfected fibroblasts (32). Furthermore, co-expression of IL-2 along with Lptn increased the antitumor response in A20 challenged mice. Expression of IP-10, a CXC chemokine, by either J558L plasmocytoma or K485 mammary carcinoma tumor cells results in the inability of those tumors to form solid masses in vivo (33). More recently, linkage of either IP-10 or MCP-3, a CC chemokine, to a non-immunogenic tumor antigen (lymphoma idiotype Ig) elicits protective immunity in response to subsequent tumor challenge (34). Work in our own laboratory has shown that MCA-205 fibrosarcoma cells modified to express the CC chemokine RANTES were unable to form solid tumors in vivo (37). Tumor growth by RANTES expressing cells were restored by depletion of either $CD8^+$ T cells or macrophages in the host animal suggesting that the antitumor response was mediated by these cell types (35). This study represented the first analysis of the functions of RANTES as produced from an in vivo source, and showed that the chemoattractant properties of this chemokine for monocytes and T cells as predicted from in vitro assays using human cells appeared to be broadly relevant in this in vivo murine model. Taken together this suggestive body of literature implicates an active role for chemokines such as RANTES and Lptn in a therapeutic setting for cancer vaccines, possibly as a mediator of T cell/APC interaction.

Recent evidence suggests that certain specific chemokines play an important role in DC priming of T cells in vivo. Mature DC, those that are most effective at priming a T cell response, and naïve T cells express the chemokine receptor CCR7 (36,37). CCR7 is the cognate receptor for both ELC (also called MIP-3β) and SLC (also called 6-Ckine), both of which are expressed predominantly in peripheral lymphoid organs (38,39). Mice deficient in expression of both SLC and ELC have diminished numbers of T cells and DC in lymph nodes (but not in spleen) and reduced trafficking of activated DC to the draining lymph node (40). Furthermore, mature DC express chemokines that can attract both naïve and recently activated T cells (41,42). It is likely that SLC (and ELC) play important roles in T cell priming by promoting DC/T cell interaction in the lymph node and spleen.

Given this background, this funded research proposal focuses on a series of studies to determine whether molecules potently and selectively chemotactic for naive and memory T cell subsets can be used in conjunction with tumor-pulsed DC to provide a highly effective means of both detecting and augmenting the immune response to breast cancer.

BODY:

The following Technical Objectives and their corresponding timelines were specified in the original funded grant application:

1. To evaluate the capacity of human dendritic cells to detect T cell specific responses to autologous breast tumor in vitro (Months 1-48).

2. To generate high, stable chemokine producer cells by the introduction and expression of the relevant genes in human fibroblast preparations (Months 1-36).

3. To determine the capacity of the combination of chemokine-secreting cells and dendritic cells pulsed with autologous breast tumor to detect, attract, and augment specific, antigen-reactive T cells in vitro (Months 12-48).

Note: All listed publications (i.e. reportable outcomes) specifically acknowledge the support of this USAMRMC grant award (DAMD17-96-1-6103) as well as its supplemental training grant (DAAG55-97-1-0239).

The progress and achievements made on the project are summarized in this Final Report as follows:

A. Brief Summary of Results Reported in the First (Year 1) Annual Report:

1.   We conducted studies to best generate "cytokine-driven" (GM-CSF, IL-4, and TNF-α) human DC obtained from peripheral blood mononuclear cells (PBMC) as measured by yield, purity, phenotype, and function as originally proposed in Technical Objective 1.

2.   We demonstrated that autologous DC could be successfully derived from PBMC of advanced breast cancer patients, which, upon antigen pulsing, could stimulate potent tetanus toxoid- and KLH-specific proliferative responses by purified T cells obtained from these same patients.

3.   The above findings considerably lessened the potential concern of a compromised immune system in advanced cancer patients (as a result of multiple chemotherapy/radiation therapy regimens that are also immunosuppressive) hampering attempts to clinically develop and utilize dendritic cell-based vaccines in our proposed breast tumor immunization approaches.

4.   We have successfully expressed three (3) distinct chemokine cDNAs (i.e. RANTES, lymphotactin, MIP-1β) constructed into the MFG-based retroviral vector as originally proposed in Technical Objective 2. Moreover, transduced fibroblasts produced significant levels of biologically active chemokine(s) by the introduced transgene(s).

B. Brief Summary of Results Reported in the Second (Year 2) Annual Report:

1.   In Technical Objective 1, we demonstrated that the addition of TNF-α to GM-CSF and IL-4 (at a critical timepoint of culture) resulted in both a significantly greater yield (at least two-fold) of DC and enhanced antigen presenting function.

2.   In Technical Objective 1, we pursued a new strategy to generate CTL that utilized known HER2/neu and CEA peptides, which have been reported to elicit specific T cell reactivity in breast cancer patients.

3.   In Technical Objective 2, we identified new chemokines (i.e. SLC/6Ckine and ELC) that have been shown to be highly selective in their recruitment of DC and/or T cell subsets (see also new data presented below).

4.   In Technical Objective 2, using a bioloistics device ("gene gun"; BioRad), we were highly successful in obtaining short-term lines of fibroblasts from breast cancer patients that secrete high levels of the transgene encoded chemokines for at least 7-10 days in culture.

5.   In response to the issues raised in the original Peer Review Panel report, we embarked on murine studies utilizing a syngeneic breast tumor (denoted MT-901).

6.   We optimized the generation of murine DC from spleen and bone marrow.

7.   We demonstrated that immunizations with tumor lysate-pulsed DC can mediate effective immune priming in vivo and can successfully treat established visceral lung metastases from the MT-901 breast carcinoma.

8.   We embarked on additional human studies with direct relevance to breast cancer patients. We were highly successful in obtaining both DC and CD34+ hematopoietic stem/progenitor cells with potent functional activity from the same leukapheresis collects from G-CSF-primed patients in sufficient numbers for the future purpose of combining peripheral blood stem cell transplantation with DC-based immunization strategies for the treatment of breast cancer.

C. Brief Summary of Results Reported in the Third (Year 3) Annual Report:

1.   In Technical Objective 1, using DC generated under fully optimized culture conditions, we were capable of reliably eliciting potent and specific T cell responses to known "model" antigens. As example, human DC derived from PBMC of an HLA-A2+ breast cancer patient cultured in GM-CSF and IL-4 in serum-free medium were able to potently stimulate autologous T cells to the defined antigen, KLH, which in all cases have surpassed the robust stimulatory capacity of an optimal concentration of the lectin, PHA.

2.   In Technical Objective 2, we focused our efforts on two new chemokines SLC and ELC. We showed that both SLC and ELC could mediate rapid and potent recruitment of naive T cells and, importantly, also DC as measured by 2-hour chemotaxis assays in vitro.

3.   In Technical Objective 2, the introduction of 6Ckine (i.e. SLC) in vivo in mice resulted in the selective recruitment into the skin site of host-derived CD3+ T cells comprised of both CD4+ and CD8+ subsets. Of importance, the number of recruited naive T cells harvested from the site of chemokine gene introduction was substantial ($>18 \times 10^6$ per centimeter diameter of excised and processed skin).

4.   We made the important discovery in our murine tumor models that the systemic administration of non-toxic doses of interleukin-2 (IL-2) could potentiate the antitumor effects of tumor-pulsed DC in vivo during both primary immunization and treatment of established tumors. Further, IL-2 could enhance CTL activity and IFN-γ production in the treated animals. These findings have resulted in our plans to initiate human clinical trials in breast cancer patients.

5.   We showed that direct intratumoral injections of "unpulsed" (i.e. not tumor antigen-loaded or charged) DC could mediate the regression of established breast tumor nodules in mice. We have further showed that antitumor efficacy appears to be correlated with the level of baseline apoptosis measured within the tumor mass before the local delivery of DC. Of

importance, tumor nodules at distant sites from the DC-injected tumor nodule also underwent regression. The immunotherapeutic effect elicited by intratumoral injections of DC in this murine breast tumor model was critically dependent upon potent activation of a host-derived T cell immune response, both locally and systemically.

6.   We also showed that pulsing DC with KLH before in vivo injection can markedly augment their antitumor effect in the MT-901 breast tumor model, which could be enhanced further by the systemic administration of IL-2.

7.   We were awarded an R01 grant from the NCI-NIH based on our data generated during this third year of the DOD project, entitled: "Experimental and Clinical Studies of the Direct Intratumoral Administration of Dendritic Cells for the Treatment of Breast Cancer". Of importance, this funded grant involves a clinical trial, entitled: "A Phase II Study of Direct Tumor Injection of KLH-Pulsed Autologous Dendritic Cells in Patients with Metastatic Breast Cancer", which received approval from our cancer center review board, IRBMED, and the FDA to proceed.

D. Brief Summary of Results Reported in the Fourth (Year 4) Annual Report:

1.   We initiated a phase II clinical trial of direct intratumoral injections of dendritic cells in advanced breast cancer patients; a second clinical trial was proposed for dendritic cells secreting the chemokine SLC.

2.   In Technical Objective 1, we evaluated the capacity of monocyte conditioned medium (MCM) to mature DC in the presence of GM-CSF and IL-4. The addition of MCM elicited maturation of human PBMC-derived DC, as measured by increases in the expression of CD83, CD80, and CD86. In every case, however, the exposure of human DC to the human recombinant trimeric form of CD40L resulted in the most pronounced increases in costimulatory molecules as well as CD83 expression on the resultant mature DC as measured by FACS analysis. The use of CD40L to mature DC has been incorporated into the production of potent human DC for some of our clinical trials.

3.   In Technical Objectives 2 and 3 (Preclinical), using our MT-901 breast tumor model in BALB/c mice, we were successful in reducing the growth of established tumor by the local delivery of the chemokine SLC. We found that intratumoral injections of SLC (on days 7-9) significantly reduced the size of the breast tumor, while subcutaneous injection of SLC at a distant site had no significant antitumor effect. To our knowledge, this finding represented the first evidence of SLC reducing breast tumor growth in vivo.

4.   We were successful in introducing the chemokine gene encoding for SLC directly into mouse and human DC rather than using primary fibroblasts. The power of this strategy allowed potent antigen presenting DC to also serve as the source of local SLC production at the vaccine site to elicit an enhanced local immune response.

5.   The in vivo injection of SLC-producing DC could not only inhibit tumor growth but could also mediate the potent migration of CD4+ and CD8+ T cells to the vaccine injection site. These data suggested that the improved adjuvanticity and antitumor effect of DC resulting from the production of SLC were due to the increased migration of T cells to the site of immunization.

6.   The data above have now positioned us to design a clinical trial in advanced breast cancer patients of SLC-producing human DC as a follow-up to our recently initiated clinical trial of intratumoral DC injections (see below).

7.   In Technical Objectives 2 and 3 (Clinical), we were awarded an R01 grant from the NCI-NIH, entitled: "Experimental and Clinical Studies of the Direct Intratumoral Administration of Dendritic Cells for the Treatment of Breast Cancer". The proposal involves translation of proposed preclinical murine studies to well-defined phase II clinical trials in patients with advanced breast cancer. A draft clinical protocol was provided with our previous (year 3) report, entitled: "A Phase II Study of Direct Tumor Injection of KLH-Pulsed Autologous Dendritic Cells in Patients with Metastatic Breast Cancer". This trial has now been initiated after receiving full approval from both our IRBMED and the FDA (under an IND). Two patients have completed treatment. No adverse, treatment-related side effects was noted. One of these two patients was featured in an ABC World News story of our work (see below). Accrural of additional patients continues.

E. Brief Summary of Results of Year 5 (no-cost extension):

1.   We demonstrated that the direct administration of DC genetically-modified to express SLC into growing tumors in mice could result in a substantial, sustained influx of T cells within the mass with only a transient increase in T cell numbers in the draining lymph node. The T cells infiltrating the tumor mass expressed the activation marker CD25 within 24 hours and developed IFN-gamma secreting function within 7 days as tumor growth was inhibited.

2.   SLC-producing DC could be retained at the tumor site with only a very small percentage trafficking to the draining lymph node.

3.   Remarkably, similar results to the above were obtained in lymphotoxin-alpha gene knock-out mice, which lack peripheral lymph nodes. Thus, T cell priming by the vaccine could occur extranodally and result in measurable antitumor effects in vivo.

4.   We evaluated the capacity of DC to effectively prime the host immune system to elicit antitumor effects in the setting of early lymphoid reconstitution after bone marrow transplantation.

5.   Remarkably, breast tumor lysate-pulsed DC could, early on during lymphopenia, prime a specific and long-lasting antitumor immune response that mediated the rejection of a lethal challenge of a breast tumor.

6.   In the therapeutic setting, we showed that breast tumor lysate-pulsed DC could also inhibit the growth of pre-existing breast tumor metastases by repetitive immunizations initiated early after bone marrow transplantation.

7.   T cells obtained from immunized animals early after bone marrow transplantation showed a substantial increase in breast tumor-specific IFN-gamma production.

8.   We demonstrated that it is possible to promote effective antitumor immunity to breast cancer in a defined lymphopenic environment through DC-based immunization; these new preclinical data will serve as the rationale for future clinical trials in breast cancer patients.

9.   Treatment of established breast tumors in bone marrow transplanted recipients could be achieved by lymph node cells primed in vivo by tumor lysate-pulsed dendritic cells.

10.   Augmentation of antitumor immunity could be achieved following bone marrow transplantation by the adoptive transfer of immune bone marrow cells from dendritic cell vaccine immunized donors.

KEY RESEARCH ACCOMPLISHMENTS:

- We have optimized the generation of dendritic cells from the peripheral blood of advanced breast cancer patients.

- We have shown that antigen-pulsed human dendritic cells can generate potent and specific T cell responses to both "standard" (KLH, TT) antigens and breast cancer-related peptides (e.g., HER2/neu) in vitro.

- We have identified chemokines (e.g., SLC/6Ckine, ELC) that can selectively recruit both naive T cells and dendritic cells.

- We have shown in preclinical animal studies that the systemic administration of non-toxic doses of IL-2 can markedly enhance the efficacy of dendritic cell-based cancer vaccines.

- We have discovered in preclinical animal studies a potentially important link between breast tumor apoptosis and susceptibility to treatment by dendritic cells.

- The chemokine SLC has potent anitumor activity in vivo in preclinical animal studies.

- We have successfully generated a recombinant adenovirus vector containing the SLC gene, which can transduce dendritic cells at high efficiency

- In preclinical animal studies, we have shown that SLC gene-modified dendritic cells can reliably produce large amounts of the chemokine and selectively recruit T cells and other dendritic cells to the site of injection in vivo.

- We have shown in preclinical animal studies that breast tumor-pulsed dendritic cells can mediate potent immunity and antitumor therapeutic efficacy in the setting of a lymphopenic state.

- We have initiated a phase II clinical trial of the direct intratumoral injection of dendritic cells into accessible nodules in patients with advanced breast cancer

REPORTABLE OUTCOMES:

A. Publications:

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29. Asavaroengchai, W., and Mulé, J.J.: Augmentation of antitumor immunity following BMT by the adoptive transfer of immune bone marrow cells from dendritic cell vaccine immunized donors (submitted).

B.   Clinical Protocol:

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1. Schott, A.F., and Mulé, J.J.: A phase II study of direct tumor injection of KLH-pulsed autologous dendritic cells in patients with metastatic breast cancer.

C.   Additional Funding Obtained Based on Work Supported by this Award:

1. U.S. Army Research Office: Augmentation Awards for Science & Engineering Research Training (AASERT): DAAG55-97-1-0239 (Principal Investigator, J.J. Mulé).

2. NCI-NIH 1 R01 CA87019-01: "Experimental and Clinical Studies of the Direct Intratumoral Administration of Dendritic Cells for the Treatment of Breast Cancer" funding period: 07/01/2000 - 06/30/2004 (Principal Investigator, J.J. Mulé).

3. NCI-NIH 2 P01 CA50327: "Cellular Vaccines for Cancer Therapy: SLC and Dendritic Cells in Antitumor Immunity and Therapy" funding period: 03/01/2001 - 02/28/2006 (Co-Principal Investigators, A.E. Chang and J.J. Mulé).

4. NCI-NIH 1 T32 CA88784-01: "Research Training in Translational Tumor Immunology" funding period: 01/01/2001 - 12/30/2006 (Principal Investigator, J.J. Mulé).

D.   Other:

The research and clinical protocol were featured on ABC World News Tonight with Peter Jennings, 9 May, 2001. "Cancer Treatment at the Cutting Edge: Hope for a Cure" (story written by Aaron Brown and produced by Nicholas Ragoush, ABC News).

CONCLUSIONS/SIGNIFICANCE:

The significance of our research lies in the potential to develop new, innovative vaccine strategies for eventual use in breast cancer patients that employs the chemokine SLC (and interleukin-2) combined with dendritic cells to both recruit/concentrate relevant immune populations at the vaccination site (by secreted SLC) as well as to activate the recruited T cells by potent presentation of tumor-associated antigens (by dendritic cells and interleukin-2). This approach may prove to be a highly effective means of both detecting and augmenting the immune response to poorly-immunogenic breast tumors that ultimately leads to tumor eradication. In addition, preclinical animal studies that have linked breast tumor apoptosis with susceptibility to treatment with dendritic cells have the potential to spawn a new generation of breast cancer clinical trials to evaluate the combination of apoptosis-inducing agents with SLC-producing dendritic cells in patients with accessible disease.

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Local Administration of Dendritic Cells Inhibits Established Breast Tumor Growth: Implications for Apoptosis-inducing Agents[1]

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ABSTRACT

Dendritic cells (DCs) can efficiently acquire foreign antigen(s) from apoptotic cells and induce MHC class I-restricted, antigen-specific CTLs. An accumulation of DCs within solid tumor masses *in situ* has been associated indirectly with a more favorable prognosis. Therefore, DCs may offer an efficient means for triggering immune responses within tumors, particularly in those masses containing significant apoptosis. We examined whether delivery of DCs could, alone, impact on the progressive growth of a tumor with a relatively high apoptotic index. We detected significant early apoptosis within the mass of a s.c. growing murine MT-901 breast carcinoma. DCs could efficiently engulf MT-901 tumor apoptotic cells *in vitro*. Intratumoral injections of syngeneic but not allogeneic DCs resulted in significant inhibition of MT-901 tumor growth. Histological examination of the tumor revealed intense mononuclear cell infiltration during and after DC injections. Tumor growth inhibition was relatively radiosensitive and dependent on host-derived CD8$^+$ T cells. The baseline level of tumor apoptosis could be increased substantially by tumor necrosis factor α administration, leading to a greater DC-mediated antitumor effect. The antitumor effect could also be enhanced by first pulsing DCs with the foreign helper protein, keyhole limpet hemocyanin, prior to intratumoral delivery and combining it with the systemic administration of interleukin 2. Splenocytes from treated animals showed heightened levels of specific CTL activity and production of cytokines. The level of *in situ* tumor apoptosis appears to play a critical role in DC-mediated antitumor effects. The potential implication of these findings in DC-based tumor therapy strategies is discussed.

INTRODUCTION

DCs[4] are potent antigen-presenting cells that can both elicit primary and boost secondary immune responses (1–3). Since their original identification by Steinman (1), much attention is now being focused on the role of DCs in eliciting antitumor immunity and in potential therapeutic applications. In this regard, DCs pulsed with defined tumor-associated peptides or proteins have been shown to elicit potent antitumor T-cell responses both *in vitro* and *in vivo* (3–5). We have reported that murine DCs can efficiently present antigens associated with whole tumor cell lysates to naive and primed T cells *in vitro* and can elicit antitumor immunity resulting in tumor regression *in vivo* (6, 7). Moreover, initial clinical trials involving DC-based immunization of patients with tumors of hematological (8) or solid tumor (9, 10)

origin have shown promise by generating antitumor T-cell reactivity as well as, in some cases, by resulting in partial and complete tumor responses. There is also indirect evidence that suggests the infiltration of solid tumor masses with greater numbers of DCs *in situ* is associated with better prognosis (reviewed in Ref. 11). Whether or not this observation directly reflects the induction of an immune response of beneficial consequence in these patients remains to be determined.

It has been shown recently that immature DCs can efficiently acquire antigen from apoptotic cells and induce MHC class I-restricted, antigen-specific CD8$^+$ CTLs (12). This finding adds additional support to the concept that DCs may play the predominant role in "cross-priming" events for the elicitation of an immune response *in vivo* (12, 13). Albert *et al.* (14) have shown further that the process of phagocytosis of apoptotic cells requires cell surface expression of $\alpha_v\beta_5$ and CD36 molecules by the immature DCs. Our recent studies have demonstrated that bone marrow-derived DCs in early culture are highly active at engulfing high-molecular-weight dextran particles *in vitro* (15). Because of these findings, it is conceivable that DCs may offer an efficient means for triggering immune responses *in situ* within tumors, particularly in those masses containing a significant baseline level of apoptotic cells.

Our preclinical and clinical therapeutic studies have involved the administration of DCs primed with whole tumor lysates (6, 7, 16, 17). In breast cancer, as an example, this approach is difficult, because only rarely has it been possible to isolate enough viable tumor cells from an individual to produce the vaccine. Thus, we have focused our efforts on designing alternative strategies to overcome this potential limitation in DC-based tumor vaccine development. In the present study, we evaluated the effect of i.t. injections of bone marrow-derived DCs on the s.c. growth of the murine MT-901 breast tumor, which we show has a prominent baseline level of early apoptosis. We demonstrate that DCs can efficiently uptake apoptotic MT-901 tumor cells and that local injections of DCs alone can result in regression of this breast tumor *in vivo*, which is dependent on host CD8$^+$ T-cell immunity. Of further importance, the *in vivo* administration of a tumor apoptosis-inducing agent, TNF-α, can enhance the therapeutic efficacy of DCs delivered locally at the site of established tumor.

MATERIALS AND METHODS

Animals. Six- to 8-week-old female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at the Animal Maintenance Facility of the University of Michigan Medical Center. The animals were used for experiments at 8–10 weeks of age.

Medium and Cytokines. CM consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 100 μg/ml streptomycin, 100 units/ml penicillin, 50 μg/ml gentamicin, and 0.5 μg/ml fungizone, all from Life Technologies, Inc. (Grand Island, NY) and 5×10^{-5} M 2-mercaptoethanol from Sigma Chemical Co. (St. Louis, MO). Recombinant cytokines were used at the following concentrations, diluted in CM: recombinant murine GM-CSF, 10 ng/ml (specific activity, $\leq 5 \times 10^6$ units/mg) from Immunex Corp. (Seattle, WA); recombinant murine IL-4, 10 ng/ml (specific activity, 2.8×10^8 units/mg) from Schering-Plough Research Institute (Kenilworth, NJ); and recombinant

human IL-2, (specific activity, 18×10^6 IU/mg) from Chiron Corp. (Emeryville, CA). Recombinant human TNF-α (specific activity, 8.2×10^6 units/mg) from Knoll AG (Ludwigshafen, Germany) was administered to tumor-bearing mice at a single dose of 6 μg i.v.

Tumors. MT-7 is a cultured murine tumor cell line derived from a dimethylbenzanthracene-induced mammary carcinoma in the BALB/c strain (18). A subline, denoted MT-901, was derived from an early *in vivo* passage of cultured MT-7 tumor injected s.c. This tumor is weakly immunogenic and expresses MHC class I (but not MHC class II) molecules. Tumors were maintained *in vitro* followed by one *in vivo* passage by s.c. injection in syngeneic mice prior to use. Tumor cell suspensions were prepared from solid tumors by enzymatic digestion in 40 ml of RPMI 1640 containing 8000 units of collagenase (type III; Sigma) for 18–24 h at 37°C, 65 rpm. The digest was then filtered over sterile 100-nylon mesh (Nytex; TETKO Inc., Briarcliff Manor, NY) and washed three times with brief incubations in HBSS (Life Technologies, Inc.). Renca is an immunogenic murine renal cell carcinoma of spontaneous origin in the BALB/c strain (19, 20).

Detection of Apoptotic Cells. Suspensions of MT-901 tumor were prepared at day 8 after the s.c. injection of 5×10^6 viable cells in BALB/c mice and were analyzed for cells undergoing apoptosis using a standard FACS assay (R&D Systems, Inc., Minneapolis, MN), which detects binding of annexin V-fluorescein and exclusion of propidium iodide (annexin V/PI assay; Refs. 12, 21, 22). Tumors of mice receiving systemic TNF-α were examined at 24 h after treatment.

In additional studies, DCs that had engulfed apoptotic tumor cells were examined by transmission electron microscopy. DCs were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 3 h at 4°C. After being washed twice in buffer, the samples were postfixed in 2% OsO4 in buffer for 1 h at room temperature. The cell pellets were washed 2 times in buffer and dehydrated in increasing concentrations of alcohol for 10 min each to final dehydration in two washes of propylene oxide. The samples were infiltrated with increasing concentrations of epon resin/propylene oxide and finally embedded in pure epon. Thin sections were obtained on an American Optical Ultracut ultramicrotome, stained with uranyl acetate and lead citrate, and viewed on a Philips 400T electron microscope.

Splenocytes. Spleen cells obtained from naive BALB/c mice were treated with ammonium chloride-potassium lysis buffer (0.83% ammonium chloride, 0.1% KHCO$_3$, and 0.004% EDTA) for 1 min to deplete erythrocytes and were washed twice with HBSS. They were then enumerated and resuspended in HBSS for injection.

Generation of Bone Marrow-derived DCs. Erythrocyte-depleted mouse bone marrow cells from flushed marrow cavities of femurs and tibias were cultured in CM supplemented with 10 ng/ml GM-CSF and 10 ng/ml IL-4 at 1×10^6 cells/ml, as described previously (15, 23). On day 3, DCs were harvested by gentle pipetting and were resuspended at 5×10^6 cells/ml in CM. Three ml of the DC suspension were overlaid onto three ml of a 14.5% (by weight) metrizamide (Sigma)-CM solution in a 15-ml centrifuge tube. The resulting gradient was centrifuged at 2000 rpm, brake off, 4°C, for 15 min. The low-density interface containing the DCs was collected by gentle pipette aspiration. The DCs were washed twice with HBSS, enumerated, and resuspended in HBSS for injection.

Antigen Pulsing of DCs. In some experiments, DCs were pulsed with KLH (M_r 350,000/400,000 subunits, endotoxin-free; Calbiochem-Naviobiochem Corp., San Diego, CA) at 50 μg/ml for 18 h.

***In Vivo* Treatment of s.c. Tumor.** BALB/c mice received 3×10^6 viable MT-901 tumor cells s.c. on day 0. In some experiments, groups of mice also first received total body irradiation with 500 rad before tumor injection. Except where specifically indicated, all of the mice were then injected on days 3, 10, 17, and 21 with 1×10^6 DCs (or normal splenocytes) in two 25-μl i.t. injections. A control group received HBSS alone. In other experiments, mice with more established MT-901 tumor received DCs i.t. on days 6, 14, and 20 in combination with i.v. TNF-α on days 5 and 13. Control mice received DC, TNF-α, or HBSS alone. The size of the tumor was assessed in a blinded, coded fashion at least twice weekly and recorded as tumor area (in mm^2) by measuring the largest perpendicular diameters with calipers, as described previously (24). Data are reported as the average tumor area $\pm$ SE.

Allogeneic DC Injection. Normal BALB/c mice were injected s.c. in the right flank with 1×10^6 MT-901 tumor cells. At day 14 after injection, the mice received i.t. injections with either 2×10^6 unpulsed allogeneic DCs in 50

μl (derived from C57BL/6), 2×10^6 unpulsed syngeneic DCs (derived from BALB/c), or HBSS. The tumor size was measured as described above.

Depletion of CD8$^+$ T cells and Treatment of s.c. Tumor. BALB/c mice were depleted of CD8$^+$ T cells by 200-μl i.v. injection of anti-CD8 (2.43, rat IgG2b) monoclonal ascites antibody (American Type Culture Collection, Rockville, MD) on days 0, 7, 14, and 21, as described previously (25). Control mice received rat IgG (Sigma) for isotype control of antibody function. The efficacy of depletion was analyzed by FACS and determined to be 99–100% effective (Ref. 25; data not shown). On day 0, all of the mice received 3×10^6 viable MT-901 tumor cells s.c. Mice receiving either anti-CD8 or rat IgG antibody were then also injected on days 3, 10, 17, and 21 with 1×10^6 DCs in two 25-μl i.t. injections. Control groups received HBSS injections. The size of the tumor was assessed in a blinded, coded fashion at least twice weekly and recorded as tumor area (in mm^2) by measuring the largest perpendicular diameters with calipers, as described previously (24). Data are reported as the average tumor area $\pm$ SE.

Treatment of s.c. Tumor with KLH-pulsed DCs. Normal BALB/c mice received 5×10^5 viable MT-901 tumor cells s.c. on day 0. The mice were then injected on days 7, 10, 13, 17, and 20 with 2×10^6 DCs in a 50-μl i.t. injection. Control groups of mice received either unpulsed DCs (2×10^6/50 μl), normal splenocytes (2×10^6/50 μl), or HBSS alone. IL-2 was given i.p. twice daily at 60,000 IU in 0.5 ml of HBSS for 2 days after each treatment. The size of the tumor was assessed, and survival was followed as recorded as the percentage of surviving animals over time (in days).

At day 120 after tumor injection, the mice that had experienced complete tumor elimination were rechallenged with 1×10^6 viable MT-901 tumor cells in the left flank and 5×10^5 Renca tumor cells in the right flank. Tumor size was then monitored as described above.

Cytotoxicity and Cytokine Assays. At day 40 after tumor injection, mice that had experienced complete tumor regression were killed to harvest the spleen. Erythrocyte-depleted splenocytes (5×10^5 cells/ml) were cultured *in vitro* with UVB-irradiated MT-901 tumor cells (2.5×10^4 cells/ml) in a 150-cm^2 flask for 5 days. On day 1, recombinant human IL-2 was added at 120 IU/ml. On day 5, the cells were collected, and dead cells were removed by density gradient. The resulting viable cells were then tested for specific cytotoxicity in a standard 4-h ^{51}Cr-release assay, as described previously (7). Percentage specific cytotoxicity was calculated as $100 \times$ [(experimental release − spontaneous release)/(maximal release − spontaneous release)]. LUs were then calculated as number of effector cells/1 $\times 10^7$ cells to achieve 20% lysis (LU$_{20}$/10^7 cells).

Aliquots of splenocytes (2×10^6 cells/ml) were also cultured for 48-h *in vitro* with 4×10^5 UVB-irradiated MT-901 or Renca tumor cells in 24-well culture plates. Culture supernatants were collected for measurements of murine IFN-γ and GM-CSF release by standard ELISA (PharMingen).

Histological Analysis. Tumor and the surrounding rim of normal skin and underlying connective tissue, at days 12, 19, and 24 after tumor injection, were excised from control and treated mice and were submitted for histological processing. The paraffin-embedded tissues were sectioned at 4 μm and stained with H&E. Slides of sectioned tissues were prepared and evaluated by a pathologist (B. J. N.).

RESULTS

Measurement of Tumor Apoptosis. We first assessed the level of baseline apoptosis in a series of s.c. growing murine tumors, including chemically induced sarcomas and a breast carcinoma. We quantitated the level of apoptosis of the dispersed solid masses by FACS analysis using the annexin V/PI assay, as described previously (12, 21, 22). In our initial screening studies, a substantial proportion (32% and 41%) of two separately harvested MT-901 breast tumors represented cells undergoing early apoptosis after s.c. injection of an initial suspension of viable single cells (Fig. 1). In contrast, the sarcomas demonstrated a relatively low baseline level of apoptosis (~4–8%) and were resistant to treatment by DCs alone administered i.t. (data not shown). These sarcomas nonetheless were inherently weakly immunogenic, similar to that of the MT-901 breast tumor, and likewise could elicit antitumor immunity *in vivo*, particularly when lysates were prepared

Fig. 1. Detection of cells undergoing apoptosis within 8-day s.c. MT-901 tumors. Tumor cell suspensions were made and analyzed by FACS for annexin V-FITC (ANN)- and propidium iodide (PI)-stained cells as described in "Materials and Methods." The two histograms shown represent tumors obtained from two separate mice. The *upper right hand quadrant*, ANN$^+$/PI$^+$; the *lower right hand quadrant*, ANN$^+$/PI$-$ staining cells; the latter is indicative of cells in the early phases of apoptosis.
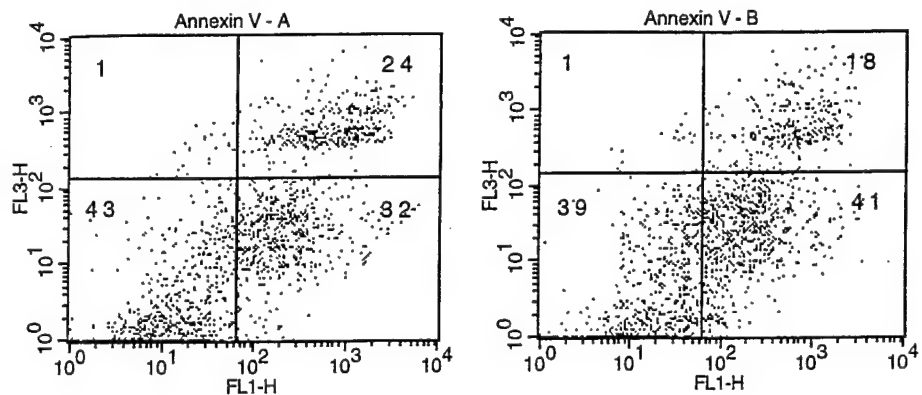
and pulsed onto bone marrow-derived DCs and used as the immunogen (7, 16).

i.t. Injections of DCs. In our earlier studies (7), immunization of mice with DCs alone failed to impact significantly on the growth of MT-901 mammary tumor located at either distant s.c. or distant pulmonary sites. To overcome the potential requirement for large numbers of DCs to first effectively traffic to and then persist within a solid tumor mass for a sufficient period to phagocytose apoptotic cells, we examined the effect of local delivery by direct i.t. injections of bone marrow-derived DCs alone. As shown in Fig. 2, bone marrow-derived DCs were highly efficient at engulfing whole, apoptotic MT-901 tumor cells *in vitro*. Mice with palpable, s.c. MT-901 mammary tumor received four courses of DCs i.t. on days 3, 10, 17, and 21 after tumor injection. As shown in Fig. 3, significant tumor growth inhibition was achieved. By day 21, tumor size in the DC-treated group averaged about 60 mm^2 compared with those of >180 mm^2 in untreated, control mice ($P < 0.01$). In a series of separate experiments, cohorts of mice that experienced complete tumor regression after DC treatment (with an overall cure rate of 20% based on 5 of 25 mice rendered completely disease-free) were rechallenged s.c. between 6 weeks and 10 months with a lethal dose (2×10^5) of viable MT-901 tumor cells. These mice all successfully rejected the rechallenge dose when compared with cohorts of naive, control animals (data not shown). The antitumor effect elicited by local DC administration could not be similarly achieved in control experiments that used normal splenocytes or allogeneic DCs. As shown in Fig. 4, murine bone marrow-derived DCs but not splenocytes obtained from syngeneic donors resulted in substantial inhibition of the growth of
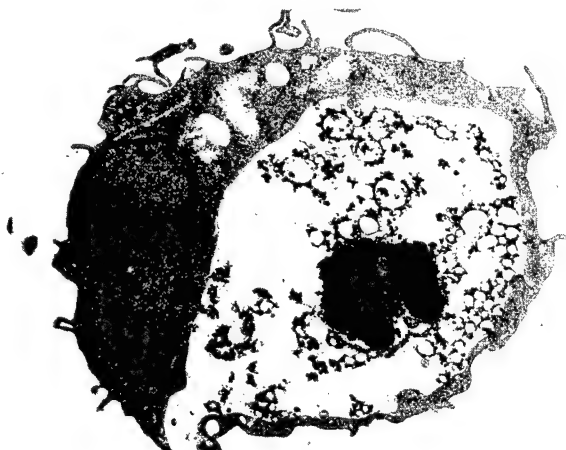

Fig. 2. Bone marrow-derived DCs efficiently engulf apoptotic MT-901 breast tumor cells. Transmission electron microscopy demonstrates the presence of an intact apoptotic tumor cell within the DC at 15 h. ×9375.

MT-901 mammary tumors after i.t. administration. By day 21, tumor size in the DC-treated group averaged about 40 mm^2 compared with those of >120 mm^2 in control mice receiving equal numbers of splenocytes ($P < 0.01$). Fig. 5 shows that the substantial antitumor effect afforded by i.t. injections of DCs was restricted to those of syngeneic but not of allogeneic origin. In other studies (not shown), allogeneic DCs were shown to efficiently engulf MT-901 apoptotic tumor cells *in vitro* to a level similar to that of syngeneic DC. Thus, although allogeneic DC were fully capable of phagocytosis, this process was not sufficient to induce tumor regression *in vivo* after i.t. administration.

Immunological Assessment of the Antitumor Effect of i.t.-administered DCs. Histological examination of skin samples was then performed on all of the tumors removed after a second (day 12), third (day 19), and fourth (day 24) i.t. administration of DCs. Fig. 6 depicts the results at day 24. At low- and high-power views, tumors from control, HBSS-injected mice were extremely large with central necrosis and extensive surrounding cohesive clusters of viable malignant cells (*i.e.*, with enlarged hyperchromatic and pleomorphic nuclei with irregular nuclear membrane and nucleoli) intermingled by polymorphonuclear leukocytes, but only rare lymphocytes (Fig. 6, *A* and *D*). At day 24, tumors removed from total body-irradiated mice that were injected with DCs i.t. exhibited prominent collections of large, viable malignant cells with focal areas of necrosis and polymorphonuclear leukocytes but with minimal mononuclear cell infiltration (Fig. 6, *B* and *E*). Tumors removed from nonirradiated mice that were injected with DCs i.t. showed early evidence of only a few viable tumor cells identified with minimal necrosis but moderate peritumoral lymphocytic infiltration. After the fourth DC injection (at day 24), only rare viable tumor cells could be identified among the extensive mononuclear cell infiltrate that generally had replaced the normal upper and deep dermis (Fig. 6, *C* and *F*). Focally, small gland formation was observed among the MT-901 tumor cells in which surrounding lymphocytes were seen in close proximity. No such evidence of differentiation by these tumor cells was seen in any of the above two control groups.

To define further the nature of the host-derived lymphoid component, we selectively depleted CD8$^+$ T cells in mice by the systemic administration of specific monoclonal antibody, as described previously (7, 25). Similar to our earlier findings reported with whole tumor lysate-pulsed DC immunizations, which demonstrated a predominant role of CD8$^+$ T cells (7), removal of this immune cell subset significantly reduced the capacity of DCs injected i.t. to inhibit the growth of the MT-901 mammary tumor (Fig. 7). In additional studies (not shown), sublethal (500 rad) total body irradiation of mice before tumor injection and DC administration was also found to eliminate the antitumor effect of DC injections; all of the treated tumors continued
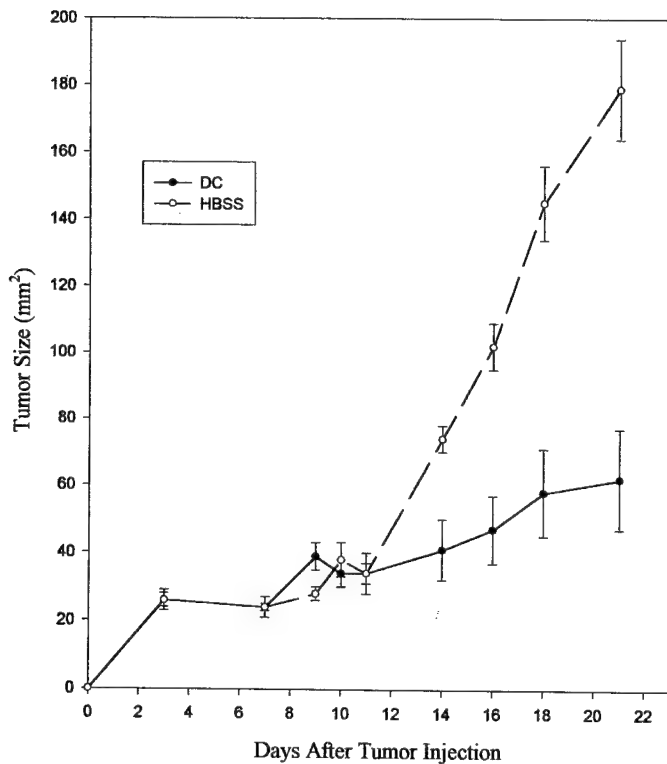
Fig. 3. Direct i.t. injections of bone marrow-derived DCs inhibit the growth of established s.c. MT-901 mammary tumor. BALB/c mice received 3×10^6 viable MT-901 tumor cells s.c. on day 0. The mice were injected on days 3, 10, 17, and 21 with 1×10^6 DCs into the tumor. A control group received HBSS alone. The size of the tumors was assessed at least twice weekly and recorded as tumor area (in mm^2) by measuring the largest perpendicular diameters. Data are reported as the average tumor area $\pm$ SE of 5 mice per group.

to grow unabated similar to those in the control mice receiving HBSS alone, which corroborated our histological findings (Fig. 6).

Enhancement of Tumor Apoptosis and DC Antitumor Effect by TNF-α. We next evaluated whether increasing the level of apoptosis within the MT-901 tumor *in vivo* could augment the antitumor efficacy of DC administered i.t., particularly against a larger tumor mass. A single i.v. injection of 6 μg of TNF-α could increase the level of apoptosis in the MT-901 tumor to >60% of the mass (Fig. 8). Mice with well-established s.c. MT-901 tumor were then treated on day 6 with DCs alone i.t. after prior systemic administration of TNF-α. As shown in Fig. 9, greater tumor growth inhibition was achieved by the combination compared with either treatment alone; 50% of mice receiving the combination therapy were rendered tumor-free.

Enhancement of DC Antitumor Effect by Foreign Helper Protein Pulsing and IL-2 Administration. We have demonstrated previously that KLH, a strongly immunogenic carrier protein, could augment the efficacy of tumor lysate- or peptide-pulsed DC immunization in mediating successful immune priming against murine tumors; this effect could be further enhanced by the systemic administration of IL-2 (27). Fig. 10 shows the results of a representative experiment; the upper and lower panels show tumor size measurements and overall survival, respectively. i.t. injections of DCs when combined with the systemic administration of IL-2 could result in substantial MT-901 tumor growth inhibition; 60% of the treated mice underwent complete tumor regression ($P < 0.05$). This antitumor effect could be enhanced further by pulsing DCs with KLH prior to i.t. injection and IL-2 administration ($P < 0.05$), which resulted in all of the treated animals experiencing complete tumor eradication and prolonged disease-free survival. All of the animals cured of established tumor were then challenged s.c. with 1×10^6 viable MT-901

tumor cells (*i.e.,* twice the dose level as that of the initial tumor challenge) in the left flank and 5×10^5 viable Renca cells in the right flank to evaluate the level and specificity of protective immunity. All of the mice were fully protected against outgrowth of the MT-901 tumor but experienced progressive growth of the irrelevant Renca tumor on the contralateral side (data not shown).

Splenocytes harvested from mice that had experienced complete tumor eradication were examined for their functional reactivity after
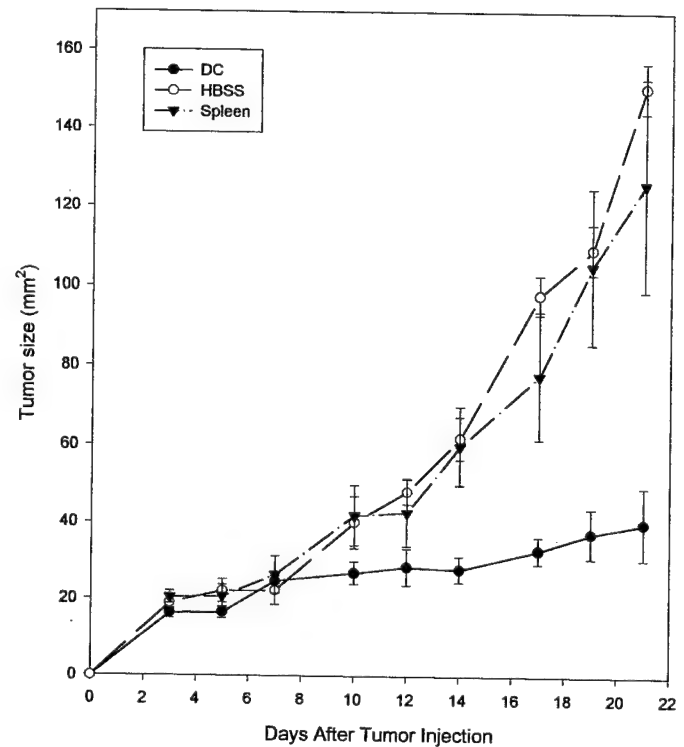

Fig. 4. Direct i.t. injections of DCs but not splenocytes inhibit the growth of established s.c. MT-901 mammary tumor. On day 0, BALB/c mice received 3×10^6 viable MT-901 tumor cells s.c. Mice were then injected on days 3, 10, 17, and 21 with 1×10^6 DCs into the tumor. Control groups received naive splenocytes alone or HBSS alone. The size of the tumors was assessed at least twice weekly and recorded as tumor area (in mm^2) by measuring the largest perpendicular diameters. Data are reported as the average tumor area $\pm$ SE of 5 mice per group.
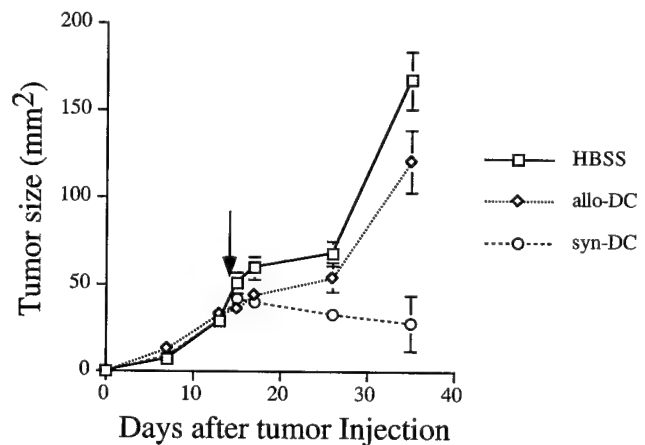

Fig. 5. Direct i.t. injection of syngeneic DCs but not allogeneic DCs inhibit the growth of established MT-901 mammary tumor. DCs were administered beginning on day 14 after s.c. tumor injection. Allogeneic DCs were generated from bone marrow cells of C57BL/6 mice, as described in "Materials and Methods." The size of the tumors was recorded as tumor area (in mm^2) by measuring the largest perpendicular diameters. Data are reported as the average tumor area $\pm$ SE of 10–11 mice per group.
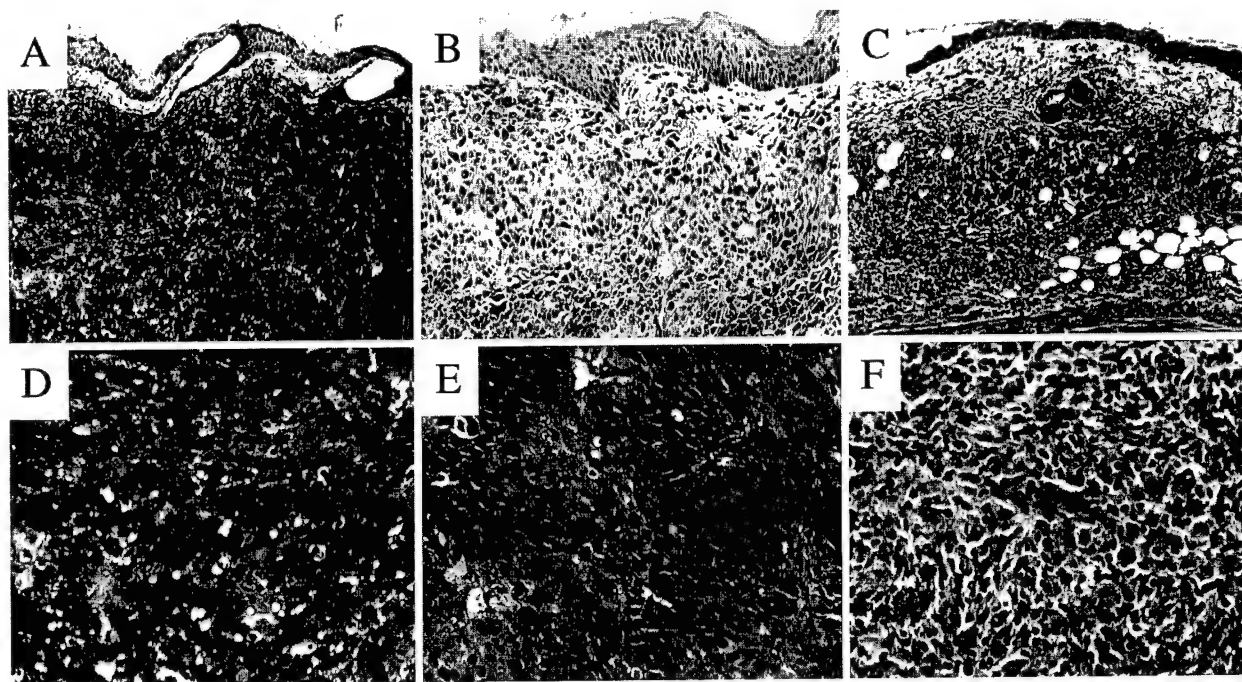
Fig. 6. Histological analysis of tissue samples removed from the skin of mice injected with MT-901 mammary tumor cells and: HBSS (*A, D*), DCs after host total body irradiation (*B, E*), or DCs alone (*C, F*). Marked host-derived mononuclear cell infiltrates are seen for the latter group. Photographs represent samples taken at day 24; mice in the DC-treated groups received four i.t. injections of 1×10^6 DCs.

in vitro restimulation. As shown in Table 1, CTLs with heightened activity against MT-901 tumor target cells could be generated from splenocytes of animals treated with KLH-pulsed DCs plus IL-2 (333 LUs) compared with DCs plus IL-2 (17 LUs) and to splenocytes from control, naive mice (<1 LU). No lysis by CTLs was detected against

Fig. 7. Host-derived CD8+ T cells participate in the *in vivo* antitumor effect of i.t. injections of DCs. BALB/c mice were depleted of CD8+ T cells or received rat IgG for isotype control of antibody function. On day 0, all of the mice received 3×10^6 viable MT-901 tumor cells s.c. Mice were then injected on days 3, 10, 17, and 21 with 1×10^6 DCs into the tumor. Control mice received HBSS alone. The size of the tumors was assessed at least twice weekly and recorded as tumor area (in mm²) by measuring the largest perpendicular diameters. Data are reported as the average tumor area ± SE of 5 mice per group.

the irrelevant, Renca tumor target (all <1 LU). The splenocytes were also examined for the production of cytokines, namely GM-CSF and IFN-γ (Fig. 11). Splenocytes isolated from MT-901 tumor-cured mice treated i.t. with KLH-pulsed DCs followed by IL-2 also produced greater amounts of GM-CSF (~2,000 pg) and IFN-γ (>11,000 pg) when specifically stimulated *in vitro* with MT-901 tumor cells. Splenocytes isolated from MT-901 tumor-cured mice treated i.t. with unpulsed DCs followed by IL-2 also produced the two cytokines, but at significantly lower amounts. As a control for tumor specificity, low-to-negligible cytokine production was observed by stimulation of the splenocytes by the irrelevant, control Renca tumor.
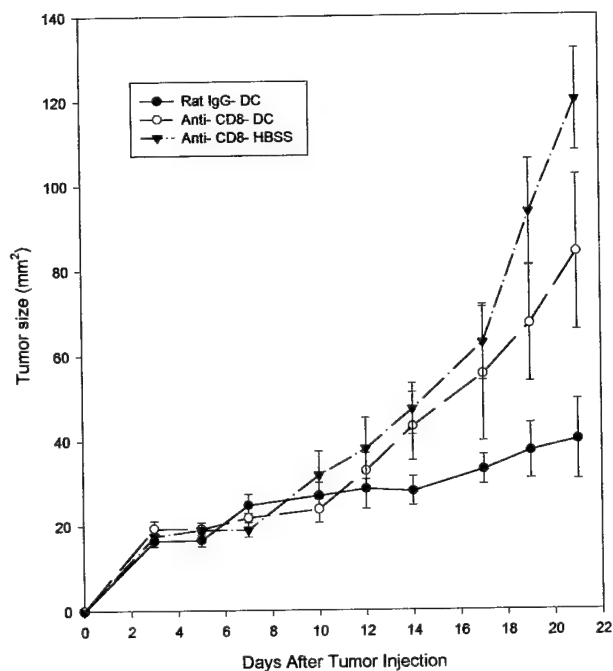
DISCUSSION

The presence of increased DC numbers within solid tumor masses has been correlated in some studies with improved prognosis (reviewed in Ref. 11). The data reported herein demonstrate that i.t. injections of DCs harvested from early (3-day) cultures of bone marrow cells in the presence of GM-CSF and IL-4 can mediate tumor growth inhibition. Similar to *in vivo* immunization studies using antigen-pulsed DCs (7), this tumor regression was dependent on host-derived CD8+ T cells and was also relatively radiosensitive. In preliminary experiments, we have also noted that the tumor growth inhibition elicited by the local administration of DCs alone but not splenocytes alone could elicit the regression of an established MT-901 breast tumor nodule distant (contralateral left flank) from the injected lesion, which again argues that the therapeutic efficacy of i.t. injections of DCs is immune mediated and is systemic in nature.

Syngeneic but not allogeneic DCs could mediate tumor regression when delivered i.t., although both sources of DCs could engulf MT-901 apoptotic tumor cells *in vitro*. Thus, the phagocytic activity of DCs to efficiently remove apoptotic tumor cells within the mass was in itself not sufficient to reduce tumor growth *in vivo*. The lack of antitumor effect by allogeneic DCs in our study is seemingly at odds with the published work of others (27, 28). In those latter studies, fusions between tumor cells and allogeneic DCs could elicit tumor

PBS

TNF-α

Fig. 8. Systemic administration of TNF-α increases the level of early apoptosis of the MT-901 breast tumor mass. BALB/c mice were injected with a single dose of 6 μg of TNF-α (*right*) or HBSS (*left*) i.v. Twenty-four h later, tumor cell suspensions were made and analyzed by FACS for annexin V-FITC (ANN)- and propidium iodide (PI)-stained cells as described in "Materials and Methods." The *upper right hand quadrant*, ANN$^+$/PI$^+$; the *lower right hand quadrant*, ANN$^+$/PI$^-$ staining cells; the latter is indicative of cells in the early phases of apoptosis.
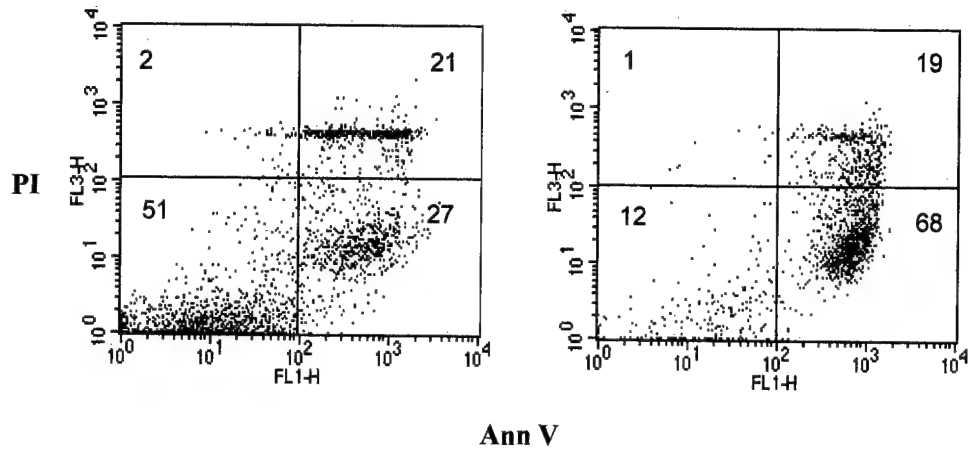
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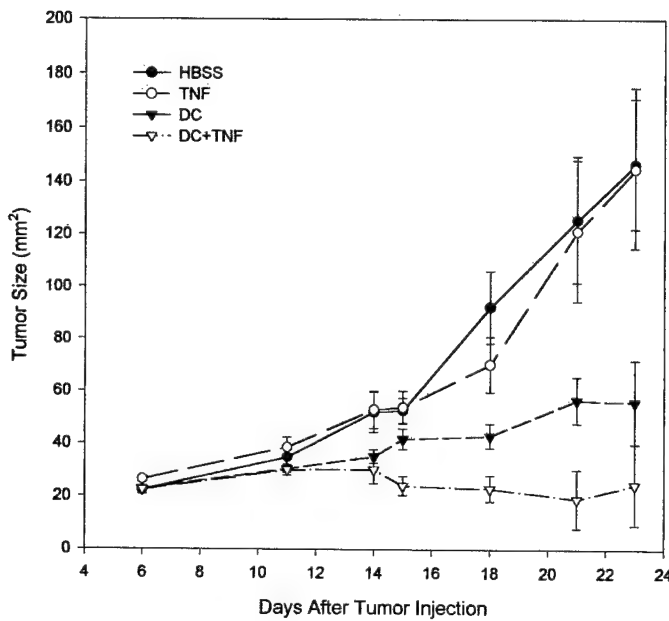
Fig. 9. Systemic administration of TNF-α enhances the antitumor effect of i.t. injections of DCs *in vivo*. BALB/c mice with day 6 s.c. tumor received DCs (1×10^6) i.t. on days 6, 14, and 20 in combination with i.v. TNF-α on days 5 and 13. Control mice received DCs, TNF-α, or HBSS alone. Tumor size was assessed twice weekly and recorded as tumor area (in mm^2) by measuring the largest perpendicular diameters with calipers. Data are reported as the average tumor area ± SE.

regression *in vivo* and could lead to the generation of MHC-restricted, tumor-specific CTLs *in vitro*. It is conceivable that heterokaryons expressing both tumor cell- and DC-derived MHC molecules after electrofusion or chemical fusion *in vitro* could explain the difference in results between these studies and ours.

We demonstrated that KLH, a strongly immunogenic carrier protein to elicit T-cell help, could enhance the antitumor effect of i.t. delivered DCs when combined with the systemic administration of IL-2. These data confirm published studies of others (29) as well as our own (27), which showed that KLH could augment by a CD4$^+$ T cell-dependent mechanism the efficacy of tumor lysate- or peptide-pulsed DCs immunization in mediating both successful immune priming toward and therapeutic rejection of tumors *in vivo* (27). Splenocytes from mice treated i.t. with KLH-pulsed DCs followed by IL-2 administration displayed heightened levels of CTL activity as well as IFN-γ and GM-CSF secretion in a tumor-specific fashion (Table 1; Fig. 11). These findings are of particular interest because both cytolytic and

noncytolytic, tumor-specific tumor-infiltrating lymphocytes have been shown to mediate potent antitumor effects *in vivo* upon adoptive transfer (30, 31).

Recent evidence has shown that immature DCs can readily acquire antigen(s) by uptake of apoptotic cells, which in turn can elicit MHC class I-restricted CTLs (12, 14). Such a process may play an important physiological role *in vivo* in the acquisition of foreign antigens *in vivo*, including those derived from tumors, virally infected and normal tissues, as well as organ transplants. Moreover, it has been shown that
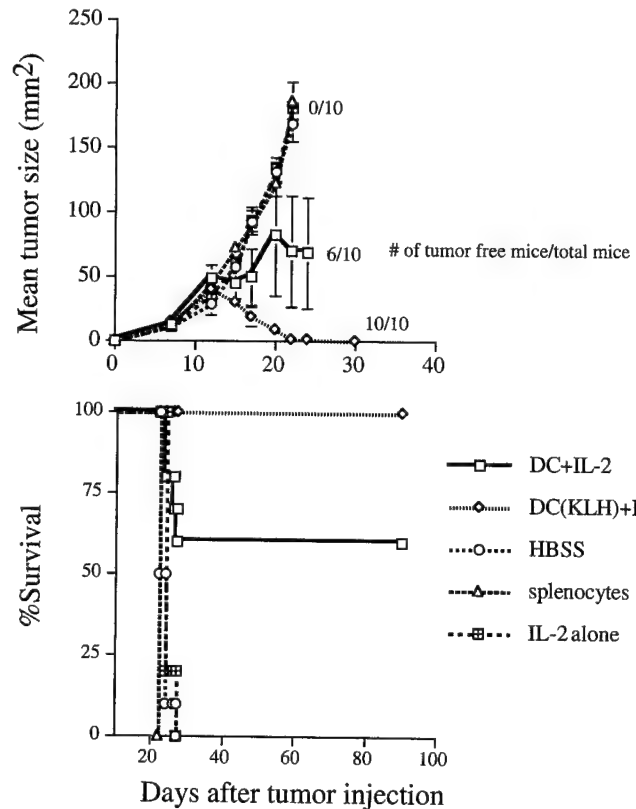

Fig. 10. KLH and IL-2 enhances the therapeutic efficacy of DCs delivered i.t. Normal BALB/c mice received 5×10^5 viable MT-901 tumor cells s.c. on day 0. The mice were then injected on days 7, 10, 13, 17, and 20 with 2×10^6 DCs in a 50-μl i.t. injection. Control groups of mice received unpulsed DCs ($2 \times 10^6/50$ μl), normal splenocytes ($2 \times 10^6/50$ μl), or HBSS alone. IL-2 was given i.p. twice daily at 60,000 IU in 0.5 ml of HBSS for 2 days after each treatment. The size of the tumor was assessed (*upper panel*), and survival was followed as recorded as the percentage of surviving animals over time (in days; *lower panel*).

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necrotic, but not apoptotic, cells can trigger maturation of DCs *in vitro* (32). Thus, it is conceivable that the balance between the levels of apoptotic *versus* necrotic cells within a tumor mass may influence the capacity of DCs to trigger an effective immune response *in situ*, which may lead to a good *versus* a poor prognosis. We had reported previously that bone marrow-derived DCs, at a relatively immature stage, could efficiently phagocytose dextran particles (15), including those of M_r 500,000. In this regard, we also showed in Fig. 2 that DCs were readily capable of efficiently engulfing intact, apoptotic MT-901 breast tumor cells.

In our current study, the MT-901 mammary tumor was found to have a prominent baseline level of cells undergoing early apoptosis within the mass. Moreover, the data of Figs. 8 and 9 demonstrated that the administration of TNF-α could mediate increased tumor apoptosis as well as enhance the antitumor effect elicited by the local delivery of DCs. We had demonstrated previously the antitumor effects of recombinant TNF-α in a variety of murine tumor models when administered alone (33, 34), or combined with chemotherapy (35) or IL-2 (36). We also showed that TNF-α mediated the antitumor effect *in vivo* by a combination of apoptotic, vascular, and immune T-cell mechanisms (33–36).

Future studies will determine whether or not other interventions that can selectively increase tumor apoptosis *in situ* and/or enhance elicited host T-cell immunity will result in more effective tumor regression by locally (or perhaps systemically) introduced *ex vivo* generated DCs alone or DCs generated directly *in situ* by the *in vivo* use of recombinant FLT-3L and CD40L (37). These efforts will be particularly important for tumors with relatively low apoptotic cell indices, which are also resistant to DC therapy alone. As examples, the systemic administration of a trimeric form of TNF-related apoptosis-inducing ligand (TRAIL) has been shown to elicit apoptosis and actively suppress certain human and murine tumors *in vivo* without demonstrable toxic side effects to normal tissues (38–40). In addition, we reported previously that the systemic administration of IL-2 could augment the antitumor effects of tumor lysate-pulsed DC vaccines (16), thus, arguing for its use in the setting of tumor apoptosis-inducing agents and local DC administration. We have also shown previously in murine tumor models that the administration of several distinct chemotherapeutic agents (*e.g.,* cyclophosphamide, 5-fluoruracil, 1,3-bis(2-chloroethyl)-1-nitrosourea, and doxorubicin) can augment the antitumor efficacy of both TNF-α (35) and IL-2 (41). Taxol (paclitaxel) can mediate tumor apoptosis directly (42, 43) and cisplatin can substantially augment the level of tumor apoptosis induced by i.t. injections of the adenovirus-p53 vector (44) in both murine and human tumors. Local delivery of a recombinant adenovirus vector encoding a wild-type p53 cDNA (Adv5-p53) has resulted in significant apoptosis of a variety of murine and human tumors experimentally (44–46) as well as more recently in Phase I clinical trials in patients with advanced non-small cell lung cancer (47) and recurrent head and neck squamous cell carcinoma (45). In laboratory studies,
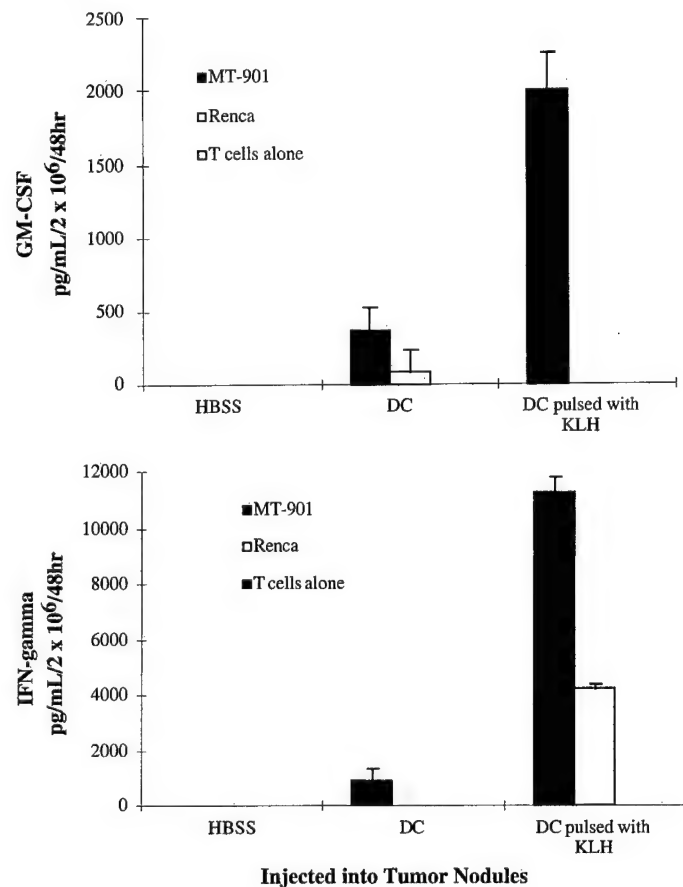

Fig. 11. i.t. delivery of KLH-pulsed DCs plus the systemic administration of IL-2 enhance specific T-cell production of cytokines. Mice were treated as described in "Materials and Methods." After tumor eradication, aliquots of splenocytes (2×10^6 cells/ml) were cultured for 48-h *in vitro* with 4×10^5 UVB-irradiated MT-901 or irrelevant Renca tumor cells in 24-well culture plates. Culture supernatants were collected for measurements of murine GM-CSF (*upper panel*)- and IFN-γ (*lower panel*)- release by standard ELISA (in pg/ml; mean ± SE of triplicate samples).

the administration of cisplatin (or VP-16) before local delivery of Adv5-p53 resulted in enhanced tumor apoptosis *in vitro* and *in vivo* as well as in enhanced antitumor effects *in vivo* (44). Indeed, in preliminary studies, we have now observed that the weakly immunogenic sarcoma MCA-207 could be rendered sensitive to therapy by i.t. injections of DCs after the induction of apoptosis within the mass by the delivery of Adv5-p53.[5]

Although we have focused on one type of DC, additional comparisons are needed. It has been suggested that the state of maturation of DCs may be important for their optimal use in immunization strategies (48, 49). Strategies that have resulted in DC maturation include the use of CD40L (37, 50–52), lipopolysaccharide (52), monocyte conditioned medium (53), and, in our own published work and that of others, TNF-α (54, 55). Also of importance to the use of DCs in our models is the discovery of DC subsets or subpopulations, which differ in their capacity to elicit antigen-specific Th1/Tc1 *versus* Th2/Tc2 immune responses (56, 57). In this regard, distinct roles of antigen-specific Th1/Tc1 and Th2/Tc2 cells may predominate during eradication of established murine tumors *in vivo* (58, 59). Moreover, it remains to be determined whether site-directed injections of immature DCs alone into apoptotic tumor-involved lymph nodes will lead to a more efficient means of eliciting both a local and systemic immune

Table 1 *CTL activity of splenocytes from mice experiencing tumor eradication by i.t. DCs*

Values are based on $LU_{20}/10^7$ cells as measured in a standard 4-hr ^{51}Cr-release assay. Spleens were harvested from BALB/c mice that had experienced MT-901 tumor eradication after i.t injections of DCs (see "Materials and Methods" for details). Splenocytes (5×10^5/ml) were cultured with irradiated MT-901 tumor cells (20:1 ratio) for 5 days. At day 1, IL-2 was added at 120 IU/ml. Cells were harvested at day 5 for testing of CTL activity.

Treatment*	Target cell lysis	
	MT-901	Renca
KLH-pulsed DCs	333	<1
DCs	17	<1
None	<1	<1

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[5] J. McLaughlin, and J. J. Mulé. A new strategy of cancer immunotherapy that involves dendritic cells and apoptosis-inducing agents, submitted for publication.

response compared with that with tumor lysate or peptide(s)-pulsed DCs injected into uninvolved lymph nodes (9).

ACKNOWLEDGMENTS

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T Cell-dependent Antitumor Immunity Mediated by Secondary Lymphoid Tissue Chemokine: Augmentation of Dendritic Cell-based Immunotherapy[1]

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ABSTRACT

Secondary lymphoid tissue chemokine (SLC) is a CC chemokine that is selective in its recruitment of naïve T cells and dendritic cells (DCs). In the lymph node, SLC is believed to play an important role in the initiation of an immune response by colocalizing naïve T cells with DC-presenting antigen. Here, we used SLC as a treatment for tumors established from the poorly immunogenic B16 melanoma. Intratumoral injections of SLC inhibited tumor growth in a CD8+, T cell-dependent manner. SLC elicited a substantial infiltration of DCs and T cells into the tumor, coincident with the antitumor response. We next used *SLC* gene-modified DCs as a treatment of established tumors. Intratumoral injections of SLC-expressing DCs resulted in tumor growth inhibition that was significantly better than either control DCs or SLC alone. Distal site immunization of tumor-bearing mice with *SLC* gene-modified DCs pulsed with tumor lysate elicited an antitumor response whereas control DCs did not. We also found that s.c. injection of lysate-pulsed DCs expressing SLC promoted the migration of T cells to the immunization site. This report demonstrates that SLC can both induce antitumor responses and enhance the antitumor immunity elicited by DCs.

INTRODUCTION

To induce an immune response against an established tumor, T cells specific for TAAs[4] must become activated, most likely by specialized cells presenting those antigens (APCs; Ref. 1). However, because most of the known TAAs are nonmutated self proteins, the T cells that can mediate tumor eradication are: (*a*) self reactive by definition; and (*b*) probably exposed to those antigens in the periphery long before any active immunotherapy is initiated (2). Therefore, the goal of a therapeutic cancer vaccine is the proper uptake and presentation of TAAs by APCs such that a specific antitumor response is initiated. Considerable effort has been made in the study of antigen types (*e.g.*, peptides, irradiated tumor cells, and gene fragments), adjuvants (*e.g.*, chemical adjuvants, cytokines, and dendritic cells), and modes of delivery (*e.g.*, DNA, pulsed DCs, and peptide/adjuvant complexes) with the explicit intent of optimizing the priming of TAA-specific T cells (3).

Because of their potent ability to stimulate T cells, particularly naïve T cells, DCs are generally conceived as the most potent members of the class of APCs (4). Based in part on current protocols that enable the generation of large numbers of DCs from peripheral blood (5, 6), DCs have been proposed as the basis of cancer vaccines. Indeed, encouraging results from preclinical and clinical studies highlight the promise of DC-based cancer immunotherapy (7–9). However, in these early studies, complete regression of tumors is not seen in the majority of patients, suggesting that modification of DC-based vaccines is required before they become a widespread treatment modality (10–12).

Genetic modification of DCs to express either tumor antigens or immunomodulatory proteins has met with success in preclinical animal models of tumor treatment (13, 14). Conceivably, DCs that process and present TAAs as transgene products present those antigens for a longer time *in vivo* than *ex vivo* pulsed DCs because of continuous gene expression and MHC loading. DCs that express cytokines may represent a longer lived or more immunostimulatory DCs, depending upon the type of cytokine gene expressed. DCs genetically modified to express GM-CSF or Lptn (a C chemokine) and pulsed with antigens induce a stronger antitumor response than control gene-modified DCs (15–17). In another treatment model, in which DCs are injected unpulsed directly into the tumor, expression of IL-12 (18) or IL-7 (19) by the DCs improves their therapeutic efficacy.

It is becoming increasingly more evident that chemokines play an integral role in the initiation of a specific immune response (20). Chemokines are a family of small secreted molecules that mediate leukocyte migration (21). One such chemokine, SLC is a CC chemokine found on high endothelial venules and within the T-cell zones of both spleen and lymph nodes (22–25). SLC is capable of recruiting both DCs and naïve T cells via the CCR7 receptor found on both cell types (26–29). Because of its expression pattern and that of its receptor, SLC has been postulated to play an important role in the priming of naïve T cells by DCs (30). Indeed, mice deficient in either SLC or CCR7 have lower steady-state levels of T cells in peripheral lymph nodes, reduced migration of hapten-primed DCs to draining nodes, and impaired immune responses to encountered antigens (31, 32).

Because both DCs and naïve T cells express CCR7, the ligand for SLC, we hypothesized that SLC could be used to initiate or enhance antitumor immunity in mice bearing established tumors. We used a mouse model of a poorly immunogeneic B16-BL6 melanoma to determine the effects of SLC on the initiation of an antitumor response. We used three distinct treatment models to assess the therapeutic efficacy of SLC: (*a*) direct intratumoral injections of recombinant SLC; (*b*) intratumoral injections of DCs genetically modified to express SLC; and (*c*) distal site immunizations of SLC expressing DCs that were pulsed with whole tumor lysate (33, 34). We used an adenovirus vector encoding SLC to modify DCs to express high levels of this chemokine. Our results show that SLC can induce a strong antitumor response that results in significant infiltration of immune effector cells into treated tumors and that genetic modification of DCs

to express SLC enhances their capacity to elicit tumor rejection *in vivo*.

MATERIALS AND METHODS

Animals. C57BL/6J (denoted B6) and BALB/c female mice, 6–8 weeks of age, were purchased from Harlan Laboratories (Indianapolis, IN) and housed at the Animal Maintenance Facility at the University of Michigan Medical Center for at least 1 week prior to use. Animals were 8 to 12 weeks of age before use in studies.

Medium and Cytokines/Chemokines. CM consisted of RPMI 1640 with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 2 mM fresh L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 50 μg/ml gentamicin, 0.5 μg/ml fungizone, and 5×10^{-5} M 2-mercaptoethanol. Recombinant murine GM-CSF (specific activity, $\geq 5 \times 10^6$ units/mg) was obtained from Immunex Corp. (Seattle, WA); recombinant murine IL-4 (2.8×10^8 units/mg) was obtained from Schering-Plough Pharmaceutical Research Institute (Kennilworth, NJ); recombinant murine SLC was obtained from Chiron Corp. (Emeryville, CA); and recombinant murine RANTES was purchased from R&D Systems (Minneapolis, MN).

Tumor Cell Lines. B16-BL6 is derived from B6 mice and is a poorly immunogenic melanoma of spontaneous origin (35). MT-901 is a subline of the MT-7 tumor cell line derived from a dimethylbenzanthrene-induced mammary carcinoma in BALB/c mice (36). Tumors were cultured *in vitro* in CM and were used before the 10th passage.

Microchemotaxis. Splenocyte responder cells were generated by gently rubbing spleens between frosted glass slides and passing over a nylon mesh filter (70 μm). RBCs were lysed, and the splenocytes were resuspended in RPMI 1640 containing 5% FCS (RPMI-FCS) and subjected to two rounds of adherence to plastic at 37°C. Nonadherent cells were resuspended to 1×10^7 cells/ml in RPMI-FCS prior to use in microchemotaxis assays. DC responders were obtained from 7-day bone marrow cultures as described below and were used at 2.5×10^6 cells/ml in RPMI-FCS. Assays were performed in 24-well plate format with 6.5-mm diameter, 5 μm pore polycarbonate Transwell insets (Costar, Cambridge, MA) in duplicate samples. SLC was added to the lower chambers at the indicated concentrations in a volume of 600 μl and incubated at 37°C for 30 min prior to addition of cells. One hundred μl of cell suspension were added to the top chamber, and the assay was carried out at 37°C in a humidified incubator with 5% CO_2. A 1:5 dilution of the cells was also directly added to the lower chamber of two wells for determination of the input amount. After 2 h, the assay was stopped by the removal of the inserts, followed by the addition of 10^4 polystyrene beads (15-μm diameter; Bangs Laboratories, Fishers, IN) to the lower chamber. Samples were stained with antibodies against CD4 and CD8 (splenocytes) or MHC II and CD86 (DCs) and counted on a FACScaliber (Becton Dickinson, San Jose, CA). In separate experiments, CD4 and CD8 cells were counterstained for expression of CD62L (all antibodies from PharMingen, San Diego, CA). The number of cells in each sample (and the input) was determined by the equation: (number of cell events/number of bead events) $\times 10^4$ beads/sample. The percentage of migration in each sample (% input) was determined by the equation: [number of cells in sample/(number of cells in input $\times$ 5)] $\times$ 100.

Treatment of Established Tumors with SLC. B6 and BALB/c mice were injected s.c. with $1-3 \times 10^5$ B16 or 1×10^6 MT901 cells (>95% viability), respectively, in the right flank. On day 6 (B16-BL6) or day 7 (MT901) when tumors were palpable, intratumoral injections of SLC (3 μg/dose, unless otherwise specified) in 50 μl of PBS + 0.05% normal mouse serum were initiated. Control groups received vehicle alone or s.c. injections of SLC in the left flank. In some experiments, B6 mice were depleted of CD4+ or CD8+ T cells by i.p. injections of 200 μl of anti-CD4 (clone GK1.5) or anti-CD8 (clone 2.43) monoclonal antibodies (both from American Type Culture Collection) 4 and 3 days before receiving the first treatment of SLC and 3, 7, and 10 days after treatment began. Control mice received isotype IgG2b (Sigma Chemical Co., St. Louis, MO). T-cell subset depletion was checked by FACS analysis and was determined to be completely effective and selective (data not shown). Tumor size was monitored twice weekly and recorded as tumor area (in mm^2) by measuring the largest perpendicular diameters with Vernier calipers. Data are reported as the average tumor area $\pm$ SE with five or more mice/group.

Generation of Bone Marrow-derived DCs. Erythrocyte-depleted bone marrow cells flushed from the femurs and tibias of B6 mice were cultured in 10 ng/ml GM-CSF and 10 ng/ml IL-4 at 1×10^6 cells/ml in CM. At day 3, fresh cytokines were added, and nonadherent cells were harvested on days 4–7 by gentle pipetting. DCs were enriched by density centrifugation over 14.5% (w/v) matrizamide (Sigma; Ref. 37). The low density population (buffy coat) was washed several times in RPMI 1640 + 2% FCS prior to use. The resulting DC population was >80% positive for coexpression of MHC II, CD11c, CD40, CD80, and CD86 (data not shown).

Tumor Harvest for Immunohistochemistry and FACS Analysis. B6 mice received 2×10^5 B16-BL6 cells s.c. in the right flank and were treated with daily intratumoral injections of 3 μg of SLC (or vehicle as control) from days 6 to 10. For immunohistochemical analysis of DCs, tumors were harvested and snap frozen in liquid N_2, and sections were analyzed by one of us (B. J. N.) for the presence of DCs with the DEC-205-specific antibody (Serotec, Raleigh, NC). DCs were counted in 10 high powered fields ($\times$40) per section (two sections/tumor) in a blinded fashion. For analysis of T-cell infiltration, B16-BL6 tumors were measured, harvested, removed of extraneous tissue, and digested for 2 h at room temperature in 1 mg/ml type IV collagenase (Sigma) with constant stirring. Digested tumors were passed over a 70 μm nylon mesh, washed once with HBSS, and resuspended in PBS + 3% BSA to approximately 1×10^6 cells/ml. Polystyrene beads (15-μm diameter) were added to the samples to achieve a concentration of 5×10^5 beads/ml. Samples were stained for the presence of CD4 and CD8 with PE-conjugated antibodies (PharMingen). Samples were analyzed by FACS with counting of 50,000 lymphocyte-sized events (based on splenocyte controls). The number of infiltrating CD4 or CD8 cells/tumor was determined by the following equation: (number of PE events/number of bead events) $\times 5 \times 10^5 \times$ cell sample volume. Because the tumors were of different sizes, the data were normalized to the tumor volume by dividing the total number of infiltrating CD4+ (or CD8+) cells by the tumor volume using the volume equation V (in mm^3) = $0.4(ab^2)$, where a is the long diameter and b is the short diameter.

Preparation of Adenoviral Vectors. aD2028#16 (Ad-SLC) carries an SLC expression cassette in its E1 region. The cassette was excised as an *Sfi*I-BspLU11I fragment from pCMVII-Amp-SLC, blunt-ended, and cloned into the *Bgl*II site of shuttle vector pD1954-*Bgl*II. The resulting plasmid contains adenoviral DNA from 0–1, 9.3–20.2, and 98.2–100 map units. This plasmid was digested with *Bsp*EI to separate the left and right ends of the adenoviral genome and recombined in BJ5183 cells (38) with Hirt prep DNA (39) prepared from mammalian cells infected with an E1-, E3-deleted adenovirus. The intact Ad-SLC genome was released from the resulting plasmid (pD2028#16) by restriction digest and transfected into C7 cells to recover virus (40, 41). pAdEasy1-GFP, containing the Ad-GFP genome, was a gift from Dr. Bert Vogelstein (42). Viruses were propagated on C7 cell monolayers and purified on CsCl gradients according to a standard protocol (43). Purified virus was dialyzed against 20 mM HEPES (pH 7.4) containing 5% sucrose, aliquoted, and frozen in a dry ice/ethanol bath (44). A_{260} was determined after particle disruption at 56°C for 10 min in 0.1% SDS, 10 mM Tris-Cl (pH 7.4), and 1 mM EDTA. Particle concentration was calculated using an extinction coefficient of 9.09×10^{13} OD/ml/cm/virion (45). Plaque assays were also performed and yielded similar vector particle:infectious unit ratios for all preparations (mean, 84 $\pm$ 11).

Genetic Modification of DCs with Adenoviral Vectors. DCs were resuspended at a concentration of 1×10^7 cells/ml in RPMI 1640 + 2% FCS and placed in a 15-ml conical tube. Virus was added at a ratio of 16,048 vector particles per DC, the suspension was mixed well, and the tube was incubated at 37°C for 2–4 h. Nine volumes of complete medium with 10 ng/ml GM-CSF and 10 ng/ml IL-4 were then added, and the cells were transferred to tissue culture dishes. Cells were incubated for 18 h at 37°C, supernatants were recovered, and the cells were purified by incubation in PBS with 3 mM EDTA and gentle scraping. Using an adenovirus encoding GFP, we determined a transfection efficiency of ~40% (data not shown). In some cases, the cells were cultured for 72 h with supernatant harvest every 24 h. The cells were washed several times in HBSS, resuspended to 5×10^6 cells/ml, and irradiated with 2000 rads prior to use.

Tumor Lysate Pulsing of Gene-modified DCs. After adenovirus infection, DCs were resuspended to 1×10^6 cells/ml in CM containing lysate from B16-BL6 cells that had been lysed by three rapid freeze/thaw reactions and spun at ~100 $\times g$ to remove cellular debris. The DCs were pulsed at a 3:1

tumor cell:DC ratio for 18 h (33, 34). After pulsing, the DCs were collected, their cultured supernatants were harvested for microchemotaxis, washed several times in HBSS, resuspended to 5×10^6 cells/ml, and irradiated with 2000 rads prior to use.

Quantitation of SLC Production by Gene-modified DCs. Because there are no currently available monoclonal antibody pairs against SLC suitable for ELISA, a microchemotaxis-based bioassay was performed to determine the amount of functional protein produced by gene-modified DCs. Supernatants from DCs infected with either Ad-GFP or Ad-SLC were added to the bottom chamber of 24-well plates in duplicate to quadruplicate samples (in some cases, a 1:2 dilution was used), and a microchemotaxis assay with splenocyte responder cells was performed as described above. Concurrently, known amounts (10, 100, 500, 1000, and 5000 ng/ml) of recombinant SLC were also added to separate wells in duplicate to generate a standard curve of SLC activity. The equation of the standard curve was generated by nonlinear regression using GraphPad Prism software. We chose a one-site binding equation $Y = (B_{max} \times X)/(K_d + X)$, where Y = % input, B_{max} = maximum migration, K_d = chemokine concentration for half maximal migration, and X = chemokine concentration. Chemokine amounts presented as ng/1 $\times 10^6$ cells in 18 or 24 h were determined from the equation derived from the standard curve for each microchemotaxis assay. The R^2 for each standard curve in nine of nine experiments was ≥ 0.92.

Treatment of Established B16-BL6 Tumors with Gene-modified DCs. B6 mice received injections s.c. of 5×10^4 B16 cells in the right flank. Treatment began on day 6 when palpable tumors of ≥ 9 mm^2 were present. DCs (5×10^5) were injected into tumors on days 6, 9, and 13. A cohort of mice were treated with daily intratumoral injections of recombinant SLC on days 6–10. As described above, tumor size was monitored twice weekly and recorded as tumor area (in mm^2) by measuring the largest perpendicular diameters with Vernier calipers. Data are reported as the average tumor area ± SE, with five or more mice/group.

Analysis of T-Cell Migration *in Vivo*. B6 mice were injected intradermally with 1×10^6 gene-modified DCs that had been pulsed with B16-BL6 tumor lysate. Skin biopsies (1.5 × 1.5 cm) including and surrounding the injection site were harvested 3 days after injection. The tissue was minced and digested for 2 h at room temperature in HBSS plus 1 mg/ml collagenase (type IV), 1500 units/ml DNase I (type IV), and 1 mg/ml hyaluronidase (type V; all from Sigma) with constant agitation. Samples were passed through nylon mesh to remove particulate matter and resuspended to approximately 1×10^6 cells/ml. Polystyrene beads were added to achieve a final concentration of 5×10^5 beads/ml. Samples were stained for the presence of T lymphocytes using PE-conjugated antibodies against CD4 and CD8. The number of infiltrating CD4 or CD8 cells/tumor was determined by the following equation: (number of PE events/number of bead events) × 5 × 10^5 × cell sample volume.

Statistical Analysis. For comparisons of treatment groups, a one-way ANOVA (followed by a Newman-Keuls post hoc test) was performed using tumor measurements taken on the last day recorded. For comparisons of two treatment groups, the Student's *t* test was performed. All statistical analysis was performed using GraphPad Prism software. Statistical significance was achieved when $P < 0.05$.

RESULTS

SLC Is Chemotactic for DC, CD4, and CD8 T Cells *in Vitro*. Prior to initiation of tumor treatment, we analyzed the effect of SLC on the migration of bone marrow-derived DCs and T cells. As seen in Fig. 1, DCs were 10–100 times more sensitive to SLC, as measured by microchemotaxis, than freshly isolated splenic T cells, consistent with previous reports (28). Among the major T-cell subsets, significantly more CD4 T-cell migration was seen in response to SLC ($P < 0.01$). In the case of both CD4 and CD8 T cells, >95% of the migrating cells were of the naïve phenotype (as measured by CD62L expression; data not shown). The DCs used in these studies were generated from 7-day bone marrow cultures in the presence of GM-CSF and IL-4, but similar chemotactic capabilities were seen from 4-day bone marrow cultures and DCs generated in the absence of IL-4 (data not shown).
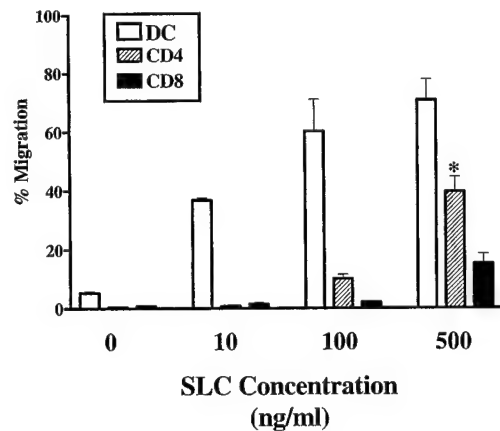

Fig. 1. Chemotactic response of DCs and T-cell subsets toward SLC. DCs (2.5×10^5/sample) derived from 7-day bone marrow cultures or splenocytes (1×10^6/sample) from B6 mice were placed in the top chamber of 6.5-mm Transwell inserts (5-μm pore size) with recombinant SLC added to the bottom chambers in the indicated concentrations. After a 2-h incubation at 37°C, 10^4 polystyrene beads were added to each well, and the samples were stained for DC markers (MHC II and CD86) or for CD4 and CD8. The number of migrating cells in each sample was calculated as described in "Materials and Methods." The migrating samples were compared with input samples that did not involve microchemotaxis, and the data are reported as the percentage of input migrating cells for DCs (□), CD4 (▨), and CD8 (■). *, $P < 0.01$ for CD4 *versus* CD8 by Student's *t* test. *Bars,* SE.

Treatment of Established Tumors with Intratumoral Injections of SLC. Because SLC was capable of attracting both DCs and naïve T cells *in vitro*, we addressed the question of whether SLC could promote an antitumor effect *in vivo*. To that end, we established s.c. tumors in B6 mice with an injection of 3×10^5 B16-BL6 melanoma cells. We began treatment 6 days after tumor challenge, at a point when palpable tumors were at least 9 mm^2. Mice were treated with daily injections of 3 μg of SLC on days 6–8, and tumor size was measured. Fig. 2*A* shows the results of one representative experiment of five performed. When SLC was administered intratumorally, B16-BL6 tumor growth was inhibited by at least 50% of that in mice treated with vehicle alone ($P < 0.05$). Intratumoral injection of SLC was necessary because tumor growth was not affected by s.c. injections of SLC in the opposite flank. To show that this antitumor effect of SLC was not strain or tumor type specific, we treated established breast tumors of the MT901 line in BALB/c mice. Again, we found that intratumoral injections of SLC (on days 7–9) inhibited growth of established MT901 tumors, whereas s.c. injection at a distant site had no significant antitumor effect ($P < 0.01$; Fig. 2*B*).

To address the dose dependence of the SLC-mediated antitumor effect, we treated 6-day established B16 tumors with three daily injections of SLC in amounts ranging from 0.1 to 25 μg. We found that 0.1 μg had little effect on tumor growth, whereas doses from 1 to 25 μg resulted in equivalent inhibition of tumor growth ($P < 0.05$; Fig. 3). In separate experiments, we found no difference between a 3-day and 5-day course of treatment or between 1 or 2 cycles of five daily injections that were separated by 2 days (data not shown). Addition of SLC to *in vitro* cultures of B16-BL6 and MT901 tumor cells had no effect on growth rates (data not shown).

To initially address the mechanism operative in the antitumor effect of SLC, we treated 6-day established B16-BL6 tumors in mice that had been depleted of either CD4 or CD8 cells 4 days prior to treatment. As seen in Fig. 4, depletion of CD8 but not CD4 cells completely eliminated the effect of SLC on tumor growth.

SLC Promotes the Recruitment of DC and T Cells to the Tumor *in Vivo*. Because SLC is chemotactic for both T cells and DCs, we hypothesized that intratumoral injections of SLC would elicit migration of these cell types to the tumor site. Mice bearing 6-day estab-
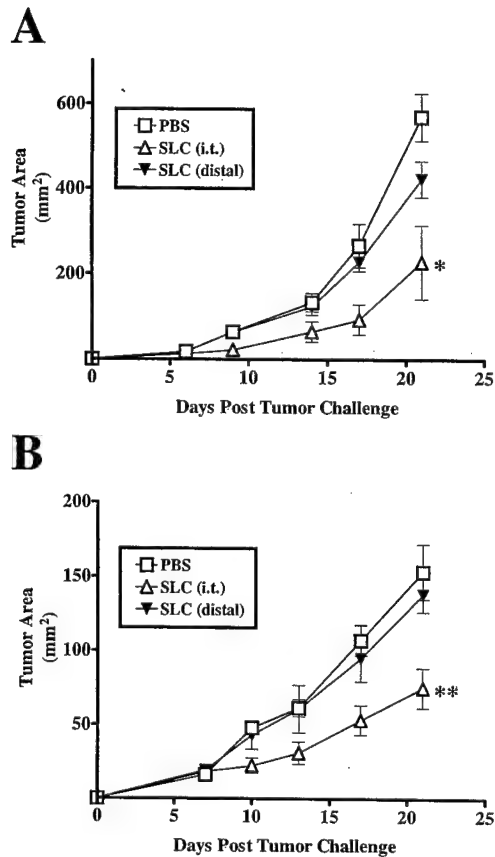
A

B

Fig. 2. Antitumor effect of SLC. *A*, B6 mice were injected with 3×10^5 B16-BL6 melanoma cells in the s.c. right flank. SLC ($\triangle$) was given intratumorally at 3 μg/dose in 50 μl of PBS (with 0.05% normal mouse serum) on days 6–8. Control groups received intratumoral injections of PBS (with 0.05% normal mouse serum) alone ($\square$) or SLC ($\blacktriangledown$) in a distal site. Tumors were measured twice weekly by measuring the longest perpendicular diameters and presented as tumor area in mm². *, $P < 0.05$ for intratumoral SLC *versus* other groups. *B*, BALB/c mice bearing 7-day s.c. tumors of the MT901 mammary adenocarcinoma (1×10^6 tumor inoculum) were treated with intratumoral injections of SLC as described above from days 7–9. Control groups and tumor measurements were as those in *A*. **, $P < 0.01$ for intratumoral SLC *versus* other groups. These data are representative of four other experiments with B16-BL6 and two other experiments with MT901, all with similar results. *Bars,* SE.

lished tumors were treated with intratumoral injections of SLC or PBS for five consecutive days. To determine the presence of infiltrating DCs, we stained frozen sections for the presence of DEC-205. Tumors from PBS-treated mice contained only rare and focal areas of tumor necrosis at 4 and 7 days after treatment; only isolated and scattered DCs were found (<1 DC/10 high-power fields). In contrast, tumors from SLC-treated mice contained more extensive and frequent zones of necrotic tumor cells (data not shown). Moreover, there were also significantly more infiltrating DCs in tumors from SLC-treated mice. Sections of SLC-treated tumors harvested 4 and 7 days after treatment began contained 6.2 ± 2.9 and 5.9 ± 1.8 DCs/10 high-power fields, respectively.

To analyze tumor infiltration by T-cell subsets, we harvested tumors on days 2 and 4 of SLC or PBS treatment. Prior to harvest, the tumor diameters were measured. After excision, tumors were enzymatically disaggregated in collagenase to obtain a single-cell suspension. We analyzed the tumor samples for the presence of CD4 and CD8 T cells by FACS analysis. To quantify the number of infiltrating lymphocytes, unlabeled polystyrene beads (15-μm diameter) were added to the samples. Because the tumors were of different sizes, we normalized the number of infiltrating cells to the tumor volume (Fig. 5). After 2 days of treatment, tumors from SLC-treated mice contained 3–5-fold more CD4 and CD8 T cells than those from PBS-

treated animals ($P < 0.05$). Significantly more infiltrating CD4 and CD8 T cells were also seen after 4 days of treatment ($P < 0.001$ and $P < 0.05$, respectively). Taken together, these data suggested that intratumoral injections of SLC could increase the number of DC and T cells within the infiltrate of s.c. tumors.

Genetic Modification of DCs to Produce SLC. Our data suggest that the presence of SLC in tumors promotes the migration of DC and T cells and inhibits tumor growth through a CD8+, T cell-dependent manner. We next determined whether alternate modes of delivery of SLC could improve its antitumor effect. In other tumor models, intratumoral injections of DCs have been shown to promote a T cell-dependent antitumor response (18, 19). We examined whether direct tumoral delivery of SLC via gene-modified DCs could improve the antitumor effect. To that end, we constructed an adenoviral vector containing the gene for SLC.

We used a microchemotaxis assay to determine the levels of SLC produced in the supernatant by gene-modified DCs. Supernatants
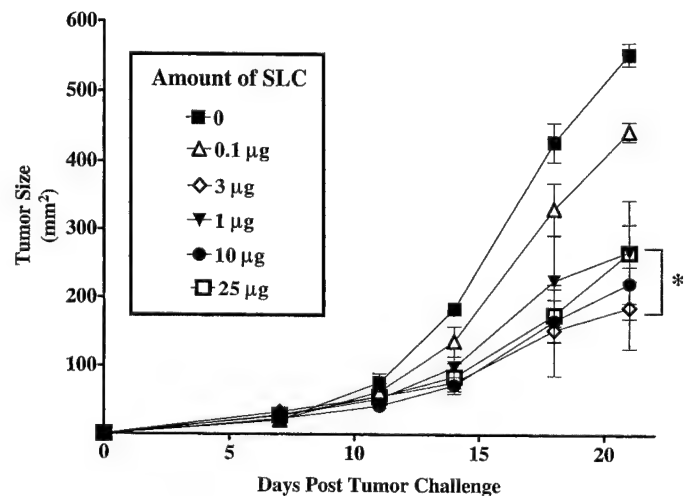

Fig. 3. Dose dependence of antitumor effect of SLC. Mice with 6-day established s.c. tumors of the B16-BL6 melanoma (1×10^5 tumor cell inoculum) were treated with three daily injections of SLC at the indicated doses. Tumors were measured twice weekly and presented as tumor size in mm². *, $P < 0.05$ for SLC doses of 1–25 μg *versus* PBS. These data are representative of two similar experiments. *Bars,* SE.
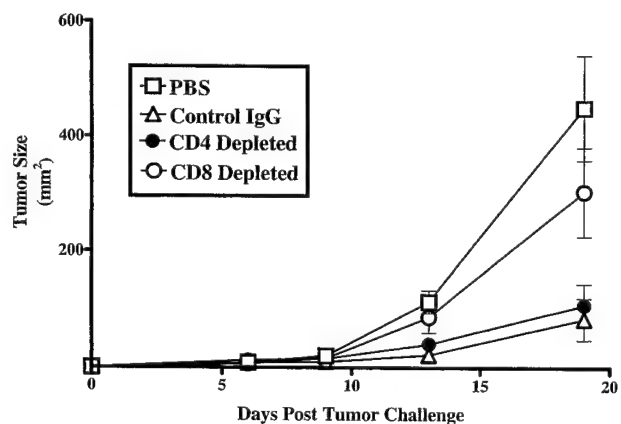

Fig. 4. Depletion of CD8+ cells abrogates the antitumor effect of SLC. B6 mice were inoculated with 1×10^5 B16 cells in the right flank. Antibodies to CD4 ($\bullet$) and CD8 ($\bigcirc$) were added 4 and 3 days before treatment as described in "Materials and Methods." Antibodies were also added 3, 7, and 10 days after treatment with SLC (3 μg/dose) began. Control mice treated with SLC ($\triangle$) or PBS ($\square$) received an equivalent amount of irrelevant isotype-matched antibody. Mice were treated from days 6 to 10, and tumor size was measured as described in Fig. 2. These data are representative of two experiments with similar results. *Bars,* SE.
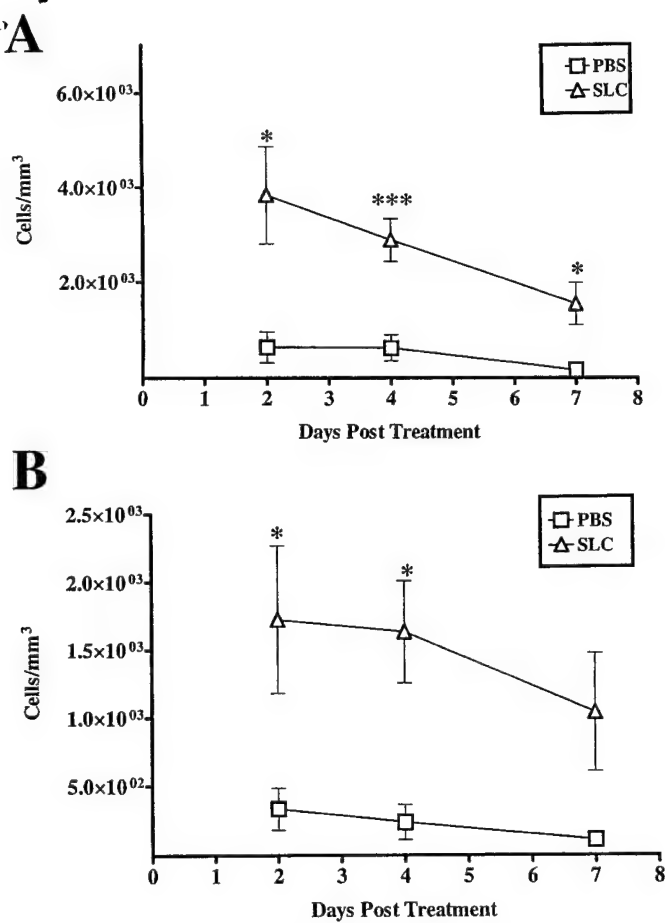
A

B

Fig. 5. Intratumoral injection of SLC elicits infiltration of host-derived T cells. A and B, tumors were harvested 2, 4, and 7 days after beginning treatment with either SLC ($\triangle$) or vehicle ($\square$), digested with collagenase, and analyzed for the presence of CD4 (A) and CD8 (B) by FACS. Quantitation of T-cell infiltration was determined by the addition of polystyrene beads (final concentration, 5×10^5/ml) and normalized to tumor volume as described in "Materials and Methods." Data are presented as the means from four to seven mice/group; bars, SE. *, $P < 0.05$; ***, $P < 0.001$ by Student's t test.

produce chemokines (46), including the CCR7 agonist ELC, these molecules did not appear to be present in large amounts in cultured supernatants.

Intratumoral Injections of SLC-expressing DCs Promote a Potent Antitumor Effect. We used *SLC* gene-modified DCs to treat 6-day established B16 tumors. Mice received three intratumoral injections of *SLC* (or *GFP*) gene-modified DCs on days 6, 9, and 13. Another cohort of mice also received daily injections of recombinant SLC for 5 consecutive days beginning on day 6. Mice receiving *GFP* gene-modified DCs showed some inhibition in tumor growth (Fig. 7), consistent with previous reports (18, 19). The antitumor effect of the GFP-expressing DCs was slightly less than that elicited by recombinant SLC. However, intratumoral injections of *SLC* gene-modified DCs elicited an antitumor effect that was significantly greater than that elicited by either the GFP-expressing DCs or SLC alone ($P < 0.01$; Fig. 7).
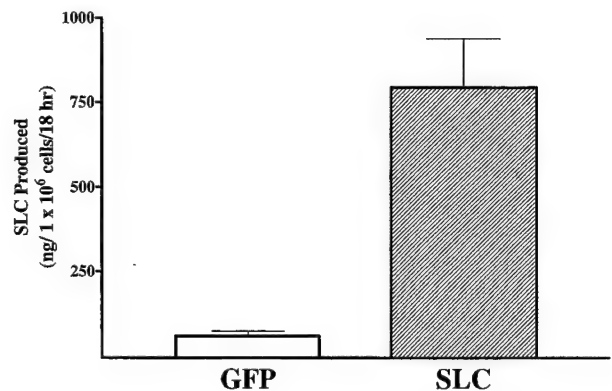

Fig. 6. Genetic modification of DCs to produce SLC. DCs were harvested from 4- to 6-day-old bone marrow cultures and infected with adenoviral vectors encoding for either SLC or GFP as described in "Materials and Methods." Supernatants were harvested 18 h after infection and were used in the bottom chamber of a 24-well plate microchemotaxis assay; CD4+ T cells from splenocytes served as responders. Recombinant SLC (10–5000 ng/ml) was used to generate a standard curve from which the effective concentration of SLC in the cultured supernatants was determined. Duplicate to quadruplicate samples were run for each supernatant tested. Data are presented as the means of the amount (in ng) of SLC produced in 18 h by 1×10^6 cells and is cumulative of nine separate infections; bars, SE.

from infected cells were used as the source of chemoattractant in microchemotaxis assays with splenic responder cells. We also performed microchemotaxis using a range (10–5000 ng/ml) of concentrations of recombinant SLC to generate a standard curve for T-cell chemotaxis. From the standard curve, we could determine the concentration of chemokine present in DC-cultured supernatants. The standard curve used in determining SLC concentrations was generated by analyzing the migration of CD4+ cells in the splenocyte responders. However, similar values were obtained using a standard curve generated from migrating CD8+ cells or by total migrating lymphocytes (data not shown). As shown in Fig. 6, which represents the data from nine separate experiments, ~750 ng of SLC were produced within 18 h by 1×10^6 *SLC* gene-modified DCs. SLC was detected in 24-h culture supernatants 3 days after infection, suggesting that gene expression endured for at least this period of time (data not shown). Genetic modification of DCs with adenoviral vectors resulted in a modest up-regulation of the T-cell costimulatory receptors CD80 and CD86 (data not shown).

The amount of chemotactic activity in cultured supernatants from *SLC* gene-modified DCs was >10 fold more than that elicited by GFP-expressing DCs (Fig. 6). Cultured supernatants from *GFP* gene-modified DCs promoted microchemotaxis of T cells to the same extent as that of cultured supernatants from the D5 variant of the B16 cell line (data not shown). This finding suggests that although DCs
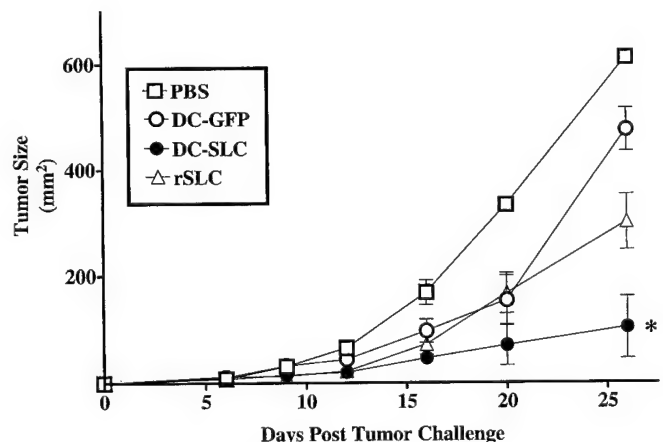

Fig. 7. Direct tumoral administration of SLC-expressing DCs inhibits growth of established tumors. Mice received injections s.c. of 5×10^4 B16 cells. DCs (5×10^5) infected 18 h before with adenovirus encoding SLC ($\bullet$) or GFP ($\bigcirc$) were given intratumorally on days 6, 9, and 13. Control mice received HBSS alone ($\square$), and another cohort received daily injections of 3 μg of recombinant SLC ($\triangle$) on days 6–10. Tumor size was measured twice weekly and is presented as tumor area in mm². This experiment was performed twice with identical results; bars, SE. *, $P < 0.01$ for DC-SLC versus SLC or DC-GFP.

Tumor Lysate-pulsed, SLC Gene-modified DCs Elicit Systemic Antitumor Immunity. We next determined whether treatment of established B16-BL6 tumors with tumor lysate-pulsed DCs could be improved by genetically modifying the DCs to express SLC. Pulsing of DCs with tumor lysate did not significantly affect the levels of SLC produced by gene-modified DCs (data not shown). We immunized mice bearing 6-day established tumors with DCs expressing SLC (or GFP) and pulsed with B16-BL6 tumor lysate contralaterally to the site of growing tumors. Fig. 8 shows that although *GFP* gene-modified DCs were ineffective in reducing the growth of s.c. tumors, SLC-expressing DCs were able to mediate a significant antitumor response. This response was dependent upon presentation of tumor antigen(s) by the DCs, because unpulsed *SLC* gene-modified DCs were unable to elicit an antitumor response when administered at a site distal from the tumor (Fig. 8).

One possible explanation for the enhanced effect of *SLC* gene modification on tumor lysate-pulsed DC immunizations is enhanced recruitment of host-derived T cells *in vivo*. Because the vast majority of DCs remain at the s.c. immunization site 24 h after injection (47, 48), it is possible that T cells would migrate to skin sites containing SLC-expressing DCs. To determine the influx of T cells into DC skin injection sites, we immunized mice s.c. with B16-BL6 lysate-pulsed DCs expressing either GFP or SLC and harvested skin samples 3 days later. After enzymatic disaggregation, we analyzed these samples for the presence of CD4+ and CD8+ cells by FACS. As shown in Fig. 9, we found that SLC-expressing DCs attracted ~2–3-fold more CD4 and CD8 T cells to the injection site at both time points tested ($P < 0.05$). These data suggest that the improved adjuvanticity of DCs resulting from expression of SLC may be attributable, in part, to increased migration of T cells to the site of immunization.

DISCUSSION

In this study, we tested the antitumor properties of SLC in three treatment regimens. Using direct tumoral administration of recombinant protein, we found that SLC could inhibit the growth of the B16 melanoma and the MT901 mammary adenocarcinoma. SLC caused a marked influx of DC, CD4+, and CD8+ T cells into the tumor mass. *In vivo* depletion of CD8+ T cells eradicated the antitumor effect of SLC. Using an adenoviral vector encoding SLC, we were able to generate SLC-expressing DCs derived from bone marrow progenitors. When given intratumorally, these *SLC* gene-modified DCs could elicit

Fig. 9. CD4+ and CD8+ T cells migrate to the injection sites containing SLC-expressing DCs. Mice received injections s.c. with 1×10^6 tumor lysate-pulsed DCs expressing either GFP (□) or SLC (▨). Three days after injection, 1.5 × 1.5-cm sections of skin were harvested, minced, and digested in collagenase, DNase I, and hyaluronidase to obtain a single cell suspension (approximately 1×10^6 cells/ml). Polystyrene beads ($C_f = 5 \times 10^5$ beads/ml) were added to enumerate migrating T cells. T-cell subsets were analyzed by FACS. Data are presented as the mean numbers of migrating cells from four (GFP) and five (SLC) mice; *bars*, SE. *, $P < 0.05$ by Student's *t* test.

an antitumor effect that was significantly better than either the recombinant protein or *GFP* gene-modified DCs. Finally, we found that *SLC* gene modification substantially improved the adjuvanticity of tumor lysate-pulsed DCs against the poorly immunogenic B16-BL6 melanoma and could attract T cells to the s.c. immunization site. Because of its poor immunogenicity, tumors of B16-BL6 origin are refractory to treatments that would ordinarily lead to tumor regression of weakly immunogenic tumor lines (17, 18), particularly when tumor burden is high (*e.g.*, 6-day s.c. tumors).

The receptor for SLC, CCR7, is expressed on both naïve T cells and DCs, suggesting that it plays an important role in T-cell activation in peripheral lymphoid organs where the chemokine is expressed (22–25). This is underscored by the fact that both SLC-deficient (*plt*) and $CCR7^{-/-}$ mice have reduced responses to antigenic stimulation (31, 32). Because we found evidence of both DC and T-cell migration in tumors treated with SLC, it is possible that the emigrated T cells are being primed in the tumor by infiltrating DCs that have taken up apoptotic/necrotic tumor cells (49). Furthermore, because mature DCs may be able to activate CD8+ T cells in the absence of CD4+ T-cell help (50), intratumoral priming of CTLs may explain why SLC treatment is efficacious, even in the absence of CD4+ cells (Fig. 4). However, we cannot rule out the possibility that mature DCs attracted to the tumor by the presence of SLC take up TAAs and migrate to the draining lymph node(s), where they activate CTLs. We are currently investigating the phenotype and function of the tumor-infiltrating T cells as well as the stimulatory capacity of emigrating DCs.

In a recent report, Sharma *et al.* (51) showed that recombinant SLC could inhibit the growth of 5-day established tumors in mice. In this particular study, SLC-mediated antitumor immunity was not elicited in either CD4- or CD8-deficient mice. The dependence upon CD4+ T cells contrasts with our data using antibody depletion (Fig. 4). These disparate results could be explained, in part, by the experimental systems used to determine subset contributions (subset-deficient mice for Sharma *et al. versus* antibody depletion here) or by the tumor models used (3LL and LC12 lung cancers *versus* B16 melanoma). Sharma *et al.* (51) also reported an increase in tumor infiltration by DC and T cells as a result of SLC treatment, which is in agreement with our data (Fig. 5).

Direct tumoral administration of other recombinant chemokines, *i.e.*, IP-10 and Mig, has been shown to inhibit tumor growth as well (52–54). In these cases, the antitumor response was mediated by the antiangiogenic properties of these chemokines. IP-10 and Mig can
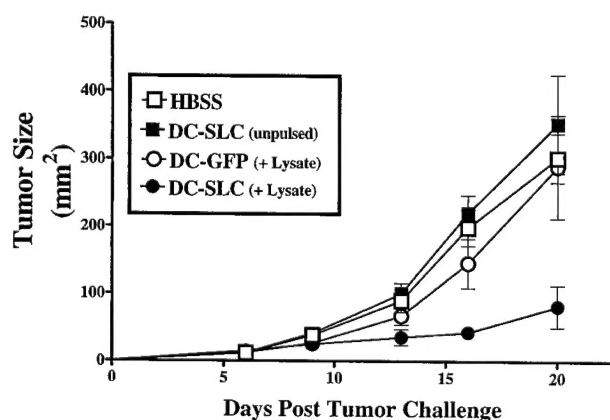

Fig. 8. Immunization with tumor lysate-pulsed, *SLC* gene-modified DCs elicits antitumor effect. Mice bearing 6-day s.c. B16-BL6 tumors in the right flank were immunized in the left flank twice on days 6 and 13 with 5×10^5 DCs that had been infected with adenovirus encoding SLC (●) or GFP (○) and pulsed for 18 h with B16 cell lysate. Control mice received injections of HBSS alone (□). Tumor size was measured as in Fig. 7. *Bars*, SE.
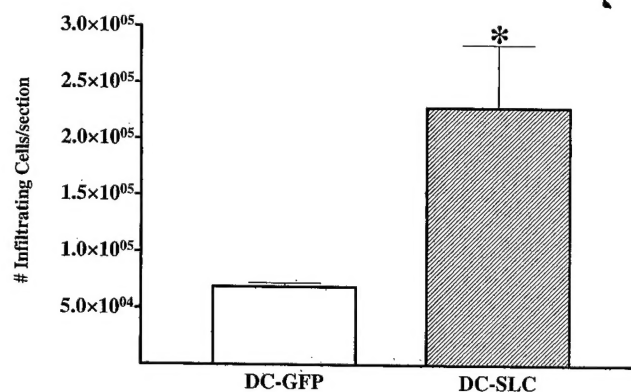
mediate the antitumor effect of IL-12 (52, 55), which may explain, in part, the increased antitumor effect elicited by *IL-12* gene-modified DCs (18). SLC has been shown to bind to CXCR3, the receptor for both IP-10 and Mig, and to exert an angiostatic activity *in vivo* (56). However, because the antitumor effect of SLC was eliminated in mice depleted of CD8+ T cells, it is unlikely that SLC mediated its antitumor effect via direct inhibition of angiogenesis. However, it remains a possibility that SLC can indirectly affect tumor vasculature via recruitment of DC and T cells that produce angiostatic agents such as IP-10 and Mig.

Antitumor therapies based on chemokine gene transfer and expression have used chemokine-transfected tumor cells, adenoviral gene delivery to tumors, and gene-modified DCs (16, 17, 57–61). Previously, we reported that tumor cells stably expressing the CXC chemokine RANTES failed to grow in immunocompetent hosts (57). Similarly to intratumoral injections of SLC, the antitumor effect elicited by RANTES-secreting tumor cells was dependent upon CD8+ T cells. However, we were unable to detect T-cell or DC migration in response to RANTES *in vitro* (data not shown). Furthermore, RANTES-secreting tumors were ineffective as a treatment against established tumors (57). More recently, it was reported that tumor cells stably expressing ELC, another ligand for CCR7, also failed to grow in immunocompetent hosts (61). In contrast to our work with SLC, the antitumor response for ELC reported by Braun *et al.* (61) was dependent upon natural killer and CD4+ cells but did not involve CD8+ cells. Lptn, a C chemokine, has been shown to enhance an antitumor effect in two gene therapy models (16, 17, 62). Immunization of tumor-bearing mice with irradiated tumors containing Lptn-secreting cells had little effect on tumor growth, but resulted in reduction of tumor growth when combined with IL-2-secreting cells (62). DCs genetically modified to express Lptn and pulsed with either peptides derived from tumor antigens or tumor RNA triggered a stronger antitumor response than control gene-modified DCs (16, 17). However, the receptor for Lptn is not expressed on naïve T cells (63), suggesting that the effect of *Lptn* gene expression depends on already activated T cells. Because CCR7 is found on naïve T cells, our results are consistent with a model in which SLC enhances the priming of naïve T cells through APCs.

Cytokine and chemokine gene-modified DCs promote stronger antitumor responses than their control gene-modified counterparts, regardless of whether the DCs are delivered intratumorally or pulsed with tumor antigens and administered at a distal site (15–19, 64). Here, we show that SLC-expressing DCs are superior to *GFP* gene-modified DCs in both treatment regimens. Gene-modified DCs express substantial amounts of SLC ($\sim$750 ng/1 $\times$ 10^6 cells/18 h), and the adenoviral vector has no detrimental effect on DC phenotype. To our knowledge, this is the first report of genetic modification of DCs to express a chemokine selective for naïve T cells. Of note, unmodified and control-modified, DC-cultured supernatants resulted in minimal migration of T cells, to an extent equivalent to those from an unmodified tumor cell line. One interpretation of these data are that although DCs express the genes for several chemokines, including ELC, they do not secrete significant amounts of the protein (46). Another possibility is that DCs cultured *in vitro* remove the secreted ELC via CCR7 expressed on their surfaces. In this model, it is possible that DCs also bound and removed the secreted SLC, but because of high expression levels, detectable amounts remained in culture.

When given intratumorally, SLC-expressing DCs reduced tumor growth of established B16 melanoma tumors to a greater extent than either DCs alone or SLC alone. Because the addition of recombinant SLC resulted in the infiltration of CD4 and CD8 T cells (along with DCs), it is likely that injection of SLC-expressing DCs also resulted

in T-cell infiltration and possible activation of T cells by the injected DCs (or by endogenous DCs attracted to the tumor by SLC). It is also possible that SLC-expressing DCs acquired TAAs and migrated to the draining lymph nodes to enhance T-cell priming. Our future studies will determine the migratory capacities of *SLC* gene-modified DCs *in vivo*. Another explanation for the enhanced effect of SLC-expressing DCs could be attributable to the bioavailability of the protein *in vivo*. Recombinant SLC was given intratumorally once daily for 5 days, whereas the DCs were given three times over the course of 7 days. Because DCs expressed high levels of SLC *in vitro* for at least 3 days, it is possible that a therapeutically effective dose of SLC in the tumor (*i.e.*, >0.1 μg) was maintained longer by the addition of SLC-expressing DCs. However, the kinetics and levels of *SLC* gene expression *in vivo* by adenovirus-infected DCs have yet to be determined.

We were also able to achieve efficacious treatment of established tumors by immunization with lysate-pulsed, SLC-expressing DCs, whereas *GFP* gene-modified DCs were ineffective as an adjuvant in this tumor model. To our knowledge, this is the first report combining chemokine gene-modification of DCs with tumor lysate pulsing to generate a therapeutically effective cancer treatment. One possible mechanism by which SLC enhanced the immunogenicity of DC-based vaccines was by the recruitment of T cells to the immunization site. It has been shown in both mice and humans that the vast majority of DCs injected s.c. remain in the injection site and do not reach the draining lymph node (47, 48). Here we showed that SLC-expressing DCs could recruit T cells to the immunization site. It is possible that the tumor lysate-pulsed DCs activated TAA-specific T cells within the migratory population locally. If indeed some T cells had become activated, it is not likely that they remained in the s.c. area for extended periods. T-cell migration to the skin in response to D5 cells expressing ELC, which binds to CCR7, occurred at 48 and 72 h after immunization but were no longer present 4 days after injection.[5] Furthermore, T cells have been shown to lose expression of CCR7 after activation (65), suggesting that, once activated, TAA-specific T cells would no longer be expected to be retained in the immunization site by the SLC-expressing DCs.

Although our results do not show unequivocally that antitumor immunity is triggered by the DCs residing in the injection site, they do suggest that SLC expression may increase the effective number of DCs (*i.e.*, those that prime naïve T cells) present in each immunization. Indeed, we have found that direct tumoral administration of SLC-secreting DCs results in tumor infiltration of large numbers of IFN-γ-secreting CD4+ and CD8+ T cells in the absence of a concomitant increase in draining lymph node cellularity.[6] Future studies using mice lacking peripheral lymph nodes should address the question of whether SLC-expressing DCs can prime an immune response without migration to lymph nodes. We have found that *SLC* gene-modified DCs are preferentially retained in the tumor compared with their control gene-modified counterparts.[6] *SLC* gene modification may obviate the need for intranodal delivery of DCs presently used in some clinical applications (12). Comparison of the route of delivery (*e.g.*, s.c. *versus* i.v. or i.p.) of *SLC* gene-modified DCs will further address the mechanisms behind the enhanced adjuvanticity of these cells. Although this study used a first-generation adenovirus, use of "gutted" adenoviral vectors (40), which can incorporate large amounts of cDNA, should allow for the gene transfer of multiple cytokine and/or chemokine genes within a single vector. These newer generation vectors are also believed to be less immunogenic than earlier versions (66), lessening the possibility of gene-modified DCs induc-

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[5] C. J. Kirk, M. Giedlin, and J. J. Mulé, unpublished results.
[6] C. J. Kirk *et al.*, manuscript in preparation.

ing antiviral immunity (67, 68). Collectively, our data demonstrate that SLC may be used as a therapeutic agent for the treatment of established tumors as both a stand-alone biotherapeutic and a gene therapy in conjunction with DC-based treatments.

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